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**HORMONAL MEDIATION OF A UNIQUE BEHAVIORAL POLYMORPHISM IN  
THE WHITE-THROATED SPARROW (*ZONOTRICHIA ALBICOLLIS*)**

By

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Bachelor of Arts, Bowdoin College, Brunswick, Maine, 2000

Dissertation

presented in partial fulfillment of the requirements  
for the degree of

Doctor of Philosophy  
in Biological Sciences, Organismal Biology and Ecology

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Hormonal Mediation of a Unique Behavioral Polymorphism in the White-throated Sparrow (*Zonotrichia albicollis*)

Chairperson: Dr. Creagh Breuner

In this body of work, I examine how testosterone (T) physiology mediates the life-history trade-off between mating effort and parental care in the White-throated Sparrow. This species exhibits a behavioral polymorphism that occurs in both sexes. White-striped (WS) morphs are more territorially aggressive, sing more frequently and seek more extra-pair copulations. Tan-striped (TS) morphs provision nestlings more frequently. Thus this species roughly illustrates the trade-off between mating effort and parental care.

I examine T physiology on three levels: plasma titres, binding globulins and response to the social environment. I ask whether levels of T correlate with morph-specific behavior and does this relationship change with stage in the nesting cycle. I found that WS males have significantly higher plasma levels of T than TS males. This difference is small, but it persisted through the parental stage of the nesting cycle. This suggests that T may mediate differences in mating effort and parental behavior in males, but is likely not the only factor. Female morphs did not differ in plasma T, thus T does not appear to play a similar role in females.

Next I ask how corticosterone binding globulin (CBG) modulates T action. I found that CBG binds over 90% of T and is an important modulator of T action in this species. However CBG capacity did not differ between morphs, nor did morphs differ in baseline levels of corticosterone (CORT, a stress hormone that competes with T for binding sites on CBG.) Therefore interactions with CBG and CORT do not affect T action differently in the morphs, and patterns of free T (T not bound to CBG) mirror patterns of total T.

Finally I investigated how T physiology responds to a change in the social environment- the establishment of a dominance relationship. WS males exhibited aggression more frequently and tended to dominate TS males. Levels of total T, CBG, CORT and free T were not predictive of future dominance status. Nor did these measures show persistent changes once the dominance relationship was established. The response of T physiology to the formation of a dominance relationship did not differ between morphs.

## DEDICATION

This dissertation is dedicated to my mother, Mary Eason Swett, whose love of the natural world I inherited and who always supported me in the pursuit of my passions. My time with you was far too short, but the lessons you taught me shape who I am today and who I will become.

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I have been blessed with wonderful friends, many of whom I met along the path to this degree. They have celebrated, commiserated, cavorted and contemplated with me along the way and made this whole process bearable. Special thanks go to B.L.W. for bringing me joy and inspiration at the end. I think you are the one I was meant to find on this crazy path. And most of all, I am grateful to my parents, David and Bobbi Swett. I wouldn't be anywhere today without your love and support. Thank you for always being in my corner- I love you.

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## INTRODUCTION

Understanding life history trade-offs is a major objective in current biological research; in the last two decades researchers investigating the mechanisms and evolution of life histories have become increasingly interested in hormones. A key question is: **how does hormone physiology mediate life history trade-offs?** Hormones are chemical signals that circulate in the bloodstream and have effects throughout the body. They act as coordinators, influencing life history traits across physiology, behavior and morphology so that each is appropriate to the life history stage. Because hormones affect multiple systems in the body simultaneously, they are said to have pleiotropic effects (Ketterson and Nolan Jr., 1999), much as genes do. This pleiotropy has important consequences for the evolution of traits. If two traits are both promoted by the same hormone, their expression may be linked. An advantageous increase in one trait, if achieved via elevation of the hormone, may lead to an increase in the linked trait, which may or may not increase fitness. Thus hormones not only mediate transitions between life history stages, but may also constrain the independent evolution of life-history traits.

Commonly, a hormone may cause an increase in one trait while causing a second trait to decrease. These instances of antagonistic pleiotropy often parallel life-history trade-offs and suggest that hormonal mechanisms may underlie these trade-offs. Studies in a broad range of taxa have implicated

hormones as mediators of life-history trade-offs. For instance, the trade-off between reproduction and survival may be hormonally mediated in both vertebrates and invertebrates. In burying beetles, juvenile hormone increases the number of eggs produced by females, but decreases their ability to survive starvation (Trumbo and Robinson, 2004). Glucocorticoids, the vertebrate stress hormones, promote behaviors that increase survival, such as foraging behavior (Angelier et al., 2007; Kitaysky et al., 2001), while decreasing investment in reproduction (Moore and Jessop, 2003; Silverin, 1998). For the most part, studies of the relationship between hormones and life-history traits have relied solely on measurements of hormone levels in the blood. But hormone systems have multiple components, and plasma titres represent just one element in the physiological system. A true understanding of the hormone-life history relationship requires a more comprehensive analysis of hormonal systems.

Hormone action is modulated at multiple levels in the body and each of these levels in turn may be responsive to changes in the environment. Clearly, hormone action will be highly dependent on the amount of hormone in the blood or plasma. But hormones do not have effects until they bind with receptors, and so the number of receptors can also have profound effects on hormone action. In addition, the receptor for a given hormone may exist in several forms each with its own effects on hormone action (Riddiford et al., 1999). The ability of hormones to access receptors may be modified by binding globulins, proteins in

the blood that bind to many hormones and prevent them from crossing capillary walls and leaving the bloodstream.

Hormonal systems are not static but rather highly responsive to the environment. It is well known that plasma hormone levels respond to environmental stimuli (Deviche et al., 2006; Hahn et al., 2004; Moshkin et al., 2002; Trumbo and Robinson, 2004), but hormone receptor number and binding globulins also respond to changes in both the abiotic and biotic environments (Bradley and Stoddart, 1992; Breuner and Orchinik, 2001; Damassa et al., 1995; Deviche et al., 2001; Perret, 1986; Pyter et al., 2007; Shaw et al., 2007). Understanding hormone action requires consideration of plasma hormone level, binding globulins, and receptors, which collectively I will refer to as “hormone physiology.”

In this body of work, I take this more comprehensive approach to examine how hormone physiology mediates a life history trade-off. The trade-off I consider here is that between mating effort and parental care. In many biparental species, individuals face a choice between investing in the offspring they have with their current mate, or spending time and energy in pursuit of additional matings. This trade-off has been well studied, particularly in birds, where many species exhibit biparental care. In birds and other vertebrates, many of the behaviors associated with mating effort are promoted by testosterone (T). These behaviors include courtship display (Salek et al., 2001), pursuit of extra-pair matings (Reed et al.,

2006) and territorial behaviors (Wingfield et al., 1987). In contrast, parental care behaviors, such as offspring defense, and incubation or feeding of offspring, frequently show a negative association with T (Stoehr and Hill, 2000; Van Roo, 2004). Thus, in many species, T appears to be an important mediator of the trade-off between mating effort and parental care.

The paradigm that T increases mating effort while decreasing parental care is not without exceptions. In species where male parental care is essential to reproductive success, elevations of plasma T do not lead to reductions in parental care (Hunt et al., 1999; Lynn et al., 2005; Steiger et al., 2006). Unlike most temperate bird species, many tropical species exhibit breeding behavior and territoriality with little elevation in T (Wingfield et al., 1991). It is hypothesized that the ability to express reproductive behaviors, territoriality etc. in the absence of high T allows these species to avoid the costs of prolonged elevations of T (Wingfield et al., 2001). Clearly, the role of T in the mating effort-parental care trade-off is more complex than initially thought.

Most studies of the relationship between T, mating effort and parental care have measured only plasma levels of T (but see (Landys et al., 2007; Lynn et al., 2007)); however T physiology, particularly in birds, is more complex. This complexity may or may not explain exceptions to the paradigm that T increases mating effort while decreasing parental effort. It most certainly must be taken into consideration when looking for relationships between T and behavior. Like

many steroids, T binds to a binding globulin in the plasma. In mammals, T is bound by a sex-steroid specific binding globulin, binding only androgens and estrogens. Birds, in contrast, have no sex steroid binding globulin and instead T is bound by corticosterone binding globulin (CBG)(Breuner et al., 2006; Deviche et al., 2001; Klukowski et al., 1997; Landys et al., 2007). Testosterone must compete with another hormone, corticosterone (CORT), for binding sites on CBG. Therefore the amount of T bound to CBG will be determined not only by the amount of CBG present (number of binding sites), but also the amount of CORT (number of competing molecules). Corticosterone is a glucocorticoid that functions in metabolism and the response to stressors. So CBG not only regulates the action of T at the plasma level, it also represents a major point of interaction between the stress hormone axis (CORT) and T. Add to this the fact that both T and CORT are immediately responsive to changes in the biotic and abiotic environment (Canoine and Gwinner, 2005; Deviche et al., 2006; Romero et al., 2006; Wingfield et al., 1990), and a very complex picture of T physiology emerges. **Here I examine how T physiology mediates the trade-off between mating effort and parental care. In the following studies I consider the effects of plasma hormone level, binding globulins, and changes in the social environment.**

Like most complex biological interactions, the relationship between a hormone and a behavior is typically noisy. A variety of research approaches have been used to aid in detecting potential associations. Relationships are most detectable

when comparing hormone measures between individuals with extreme values of the behavioral trait. But behavior and other traits are usually normally distributed, making individuals with extreme trait values rare and hard to sample. One way to circumvent this is to compare hormone measures between species that vary in the behavior of interest. Other studies compare hormone levels between males and females and correlate these differences with a difference in behavior between the sexes. While inter-species and inter-sex comparisons have been informative, confounding factors are inherent to these approaches and may complicate interpretation of the results of these studies. A third approach is to experimentally manipulate hormone levels and measure effects on behavior and other traits. This approach, called phenotypic engineering (Ketterson et al., 1996), has proven very productive, but is not without its own drawbacks. For instance, some aspects of hormone physiology, such as CBG or receptor number, may be impossible to manipulate directly with currently available pharmaceutical tools.

Here I use yet another approach and examine the relationship between T physiology and the mating effort/parental care trade-off by taking advantage of a behaviorally polymorphic species. In these species, there is exaggerated behavioral variation within a sex leading to a bimodal (or in some species trimodal) distribution of a behavioral trait within a sex. This allows for powerful comparisons between individuals that exhibit distinct behavioral phenotypes but are still members of the same species and sex. Behaviorally polymorphic

species allow us to avoid introducing many confounding factors and exploit a “natural” phenotypic engineering experiment.

Behaviorally polymorphic species have been used profitably to explore the hormonal mechanisms of male alternative reproductive strategies. Most of these species exhibit a polymorphism only within the male sex. Morphs often take the form of a territorial morph that performs more sexual displays and exhibits male secondary sex characteristics, and a sneaker or satellite male who displays less and may mimic female morphology (Bass and Andersen, 1991; Sinervo and Lively, 1996; Thompson and Moore, 1992). However, most endocrine studies of behaviorally polymorphic species measure only hormone levels in the plasma and do not consider other aspects of hormone physiology. A recent review by Knapp (Knapp, 2004), highlights more recent investigations that consider the role of binding globulins and other factors that may modulate hormone action.

Knapp also emphasizes the need to consider interactions between different hormone axes, as well as the axes’ interactions with the environment in future studies of behaviorally polymorphic species. These recommendations are in agreement with the more comprehensive analysis of hormone physiology that I advocate above.

In the following three chapters, I investigate hormone physiology in a behaviorally polymorphic species that is uniquely suited for studying the trade-off between mating effort and parental care. The white-throated sparrow is a migratory

songbird that breeds in the northeastern United States and across the Canadian shield, and winters in the southeastern U.S. These sparrows are typically socially monogamous and exhibit biparental care with males assisting with the feeding of nestlings and fledglings (Falls and Kopachena, 1994). Unlike other polymorphic species described above, white-throated sparrow morphs can be roughly classified as “more aggressive” and “more parental.” Birds with white-stripes on their crown (WS) respond more aggressively to territorial intrusions (Kopachena and Falls, 1993a), sing more frequently (Falls and Kopachena, 1994) and may pursue more extra-pair copulations (Tuttle, 2003). Tan-striped birds (TS), in contrast, provision nestlings at a higher rate (Kopachena and Falls, 1993). This species is also advantageous for the present study because each morph occurs in both sexes. Morph-type is determined by a chromosomal inversion on the second somatic chromosome (Thornycroft, 1967; Thornycroft, 1975) and is not-sex linked. WS females provision nestlings less than TS females (Kopachena and Falls, 1993), solicit copulations more frequently (Tuttle, 2003) and respond more aggressively to territorial intrusions (Kopachena and Falls, 1993a). In the white-throated sparrow, morph-types illustrate the mating-effort/parental care trade-off providing us with an ideal opportunity to study hormonal mediation of this trade-off in both sexes.

This dissertation addresses how T physiology may mediate the trade-off between mating effort and parental care in the White-throated Sparrow. **Specifically, does T physiology mediate morph-specific patterns in territorial aggression**



**and parental care in this species?** First, I ask whether plasma levels of T correlate with morph-specific behavior during the breeding season and how this relationship might change with stage in the nesting cycle. Second, I examine how T action is modulated by CBG, as well as how interactions between T, CBG and CORT may differ between the morphs. Finally, I examine how all of these parameters respond to a change in the social environment. These studies represent a comprehensive analysis of the hormonal mediation of a life history trade-off. Understanding the relationship between hormone physiology and life history helps us understand not only the mechanisms of life history traits, but informs our understanding of life histories at all levels of analysis.

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## Chapter 1

### PLASMA TESTOSTERONE CORRELATES WITH MORPH-TYPE ACROSS BREEDING SUB-STAGES IN MALE WHITE-THROATED SPARROWS

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**Keywords:** *behavioral polymorphism, aggression, testosterone, gonadotropin  
releasing hormone*

## Abstract

The White-throated Sparrow (*Zonotrichia albicollis*) exhibits a genetic polymorphism that affects plumage and behavior in both sexes. Birds belonging to the white-striped morph are more territorially aggressive while tan-striped morphs provision nestlings at a higher rate. We investigated testosterone physiology in this species in an effort to understand hormonal mechanisms for differences in aggression and parental care observed between the morphs.

1. We found small but significant differences in plasma testosterone between white-striped and tan-striped males over the course of the breeding season. This difference correlates with previously observed differences in aggressive behavior and suggests that testosterone may play a part in mediating these differences. Testosterone remained higher in white-striped males relative to tan striped males while males were provisioning nestlings and fledglings. Thus, testosterone may also be contributing to the relatively reduced levels of parental care exhibited by white-striped males.

2. We found no difference in plasma testosterone between white-striped and tan-striped females, suggesting that testosterone does not mediate differences in aggression between female morphs.

3. Injection with exogenous gonadotropin releasing hormone led to greater testosterone secretion in both males and females, but did not differ by morph. Therefore we conclude that differences in plasma testosterone between the morphs are due to differences in testosterone regulation upstream of the pituitary.

### Introduction

The relationship between hormones and behavior, like most biological relationships, is noisy. Correlations between a hormone and a behavior will be most easily detected when comparing individuals with extreme values of a behavioral trait. However, within a species or sex, behavioral traits are typically normally distributed and thus these “extreme” individuals will be rare. In order to compare distinct behavioral phenotypes, researchers often compare individuals of different species. This approach has been informative, but involves many confounding factors such as differences in species ecology. Another approach that has proved successful is phenotypic engineering (Reed et al., 2006) in which treatment with exogenous hormones is used to exaggerate differences between endocrine phenotypes within a natural range.

Behaviorally polymorphic species offer a natural case of phenotypic engineering providing exaggerated, often bimodal distributions of a behavior trait within a single population. This allows us to compare individuals that exhibit distinct

behavioral phenotypes, and makes relationships between hormones and behavior easier to detect. Thus, behaviorally polymorphic species are a powerful tool for studying the endocrine bases of behavior. Many species across a variety of taxa exhibit two or more behavioral morphs within a sex. In most cases, the polymorphism is only exhibited by the male sex and these male morphs exhibit alternative reproductive strategies. Commonly one morph will be a territorial, aggressive phenotype, while the other morph is less aggressive and employs a “sneaker” or a “satellite” strategy (Brantley et al., 1993; Lank et al., 1995; Sinervo and Lively, 1996). These species have been used very successfully to study the hormonal and neural mechanisms of courtship behaviors and territorial aggression.

The White-throated Sparrow (*Zonotrichia albicollis*) exhibits a very different behavioral polymorphism that is distinguished by two important features. First, morph types may be roughly classified as a territorially “aggressive” morph that expends more effort in pursuit of extra-pair matings and a “parental” morph. White-striped (WS) birds are more aggressive in response to simulated territorial intrusion (Kopachena and Falls, 1993b), they are estimated to have higher rates of extra-pair copulation (based on rates of intrusion into neighboring territories (Tuttle, 2003) and they sing more frequently than do tan-striped (TS) birds (Falls and Kopachena, 1994b). Tan-striped birds, the “parental” morph, provision nestlings at a higher rate than their WS counterparts (Kopachena and Falls, 1993). The “aggressive” and “parental” morphs represent a very different



dichotomy than the more common “territorial” morphs and “sneaker” morphs. Morph types in this sparrow illustrate what is thought to be a fundamental trade-off between mating effort and parental care (Trivers, 1972). Thus, this species presents us with an ideal opportunity to study hormonal mechanisms of this putative trade-off. Second, morph type is determined by a pericentric inversion on the second somatic chromosome, and is not sex-linked (Thornycroft, 1966; Thornycroft, 1975). Thus, females exhibit both morph-types, in contrast to most other polymorphic species in which only males exhibit the polymorphism. This presents a unique opportunity to study endocrine bases of aggressive and parental behavior in females, as well as males.

Data from many taxa suggest that testosterone (T) may mediate the trade-off between mating effort and parental care that is illustrated by the morphs (Fleming et al., 2002; Reburn and Wynne-Edwards, 1999; Wingfield et al., 1990b; Young et al., 2005). Testosterone and other androgen levels are positively associated with the breeding season aggressive and sexual behaviors that are more pronounced in the WS morph (Balthazart, 1983; Schwabl, 1991; Wingfield et al., 1987a). In contrast, experimentally elevated T often leads to a reduction in parental care behavior (Schoech et al., 1998; Schwagmeyer et al., 2005; Van Roo, 2004) and males that provide parental care usually show reduced T levels when they enter the parental stage of the nesting cycle (Wingfield et al., 1987b). Thus WS birds, both male and female, would have higher levels of plasma T relative to TS birds. If T is also mediating differences in parental care, we expect

that the difference in T levels will persist into the parental stage of the nesting cycle. In addition to comparing plasma T levels, we also investigated how differences in T between the morphs might be generated. We compared the sensitivity of the hypothalamic-pituitary-gonadal axis (which regulates the secretion of T) between the morphs. We expected that higher levels of plasma T would be associated with differences in testosterone regulation at the level of the pituitary or gonad.

## Materials and Methods

### Study Species

The White-throated sparrow is a migratory songbird that breeds in the northeastern United States and Canada and winters in the southeast U.S. This species is primarily socially monogamous though rarely males (usually WS) will attempt polygamy. Males assist with feeding of nestlings and fledglings but do not incubate (Falls and Kopachena, 1994a). Sparrows in our study population may rear two broods per season and will reneest 3 or more times if nests are depredated (personal observation.)

### Collection of Field Samples

Breeding sparrows were observed and captured on forested property owned and/or managed by the Northwoods Stewardship Center in East Charleston, Orleans County, Vermont. Samples were collected between 22 May and 29 July

2003, 2 May and 23 July 2004, and 21 April and 10 July 2005. A total of 53 males (20 TS and 33 WS) and 37 females (13 WS and 24 TS) were sampled. Sparrows were captured in seed-baited Potter traps or mist-nets. Mist-nets were either placed near the nest, or birds were attracted to the net using playbacks of conspecific songs. Blood samples were collected by venipuncture of the alar vein and blood was drawn into a heparinized microhematocrit tube via capillary action. Samples used to measure T were collected within 10 minutes of the bird contacting the net or the fieldworker approaching the potter trap (3 out of 139 were collected within 15 minutes) so as to minimize the effect of stress on T levels (Lance et al., 2004; Moore et al., 2000). All birds were banded with a U.S. Fish and Wildlife numbered band as well as a unique combination of colored plastic leg bands allowing birds to be identified visually at a distance. Nests were located and monitored for as many individuals as possible in order to determine the stage in the nesting cycle at which each sample was taken. Blood samples were kept on ice while in the field (up to four hours.) Each sample was then centrifuged and plasma drawn off using a Hamilton syringe. Plasma was kept frozen at approximately -20 °C until it could be assayed.

#### GnRH Challenge

Lab: Wintering sparrows were captured in Travis County, Texas in seed-baited Potter traps or seed-baited mist nets. Birds were housed in individual 13" x 15" x 17" cages in captivity and were photo-stimulated (14L:10D) for 3 weeks to bring birds into pseudo-breeding condition prior to the start of the experiment.

We followed a GnRH challenge protocol previously used in dark-eyed juncos, a closely related and similarly sized species (Jawor et al., 2006). A blood sample was taken from each individual via venipuncture of the alar vein. Birds were then given an intramuscular injection in the pectoralis muscle of either 50 $\mu$ L GnRH diluted in saline (25ng/ $\mu$ L) or 50 $\mu$ L saline as a control. They were then held in a cloth bag for 30 minutes at which point a second blood sample was taken. One week later the experiment was repeated as described except that individuals that had received the GnRH treatment now received a control injection and vice versa.

Field: The GnRH challenge experiment was repeated using free-living breeding birds, but was limited to males only. Male sparrows were caught at the field site in Orleans County, VT and were bled, injected and bled again as described above except that separate sets of birds received the GnRH and saline treatments. All birds included in this experiment were caught between May 10 and May 27, 2005, prior to the first egg of the season. This early in the breeding season, most of the birds are relatively synchronized in their nesting attempts and we expected reduced variation in baseline T levels between males.

Statistical Analyses: Values of plasma testosterone in all experiments (except the field GnRH trial) were not normally distributed (positively skewed) and were log transformed ( $\log(T+1)$  or  $\ln T$ ) to correct for this. During the course of the field study 53 males (33 WS, 20 TS) and 37 females (13 WS, 24 TS) were sampled. Some individuals were sampled more than once over the course of the

field study, but no individual was bled more than once in any 7 day period to minimize the physiological effects of sampling. A total of 94 samples were collected from males with a mean of 1.8 samples per individual (range 1-7). 43 female samples were collected with a mean of 1.4 samples per female (range 1-3). Field samples obtained from males and females were analyzed separately. T levels were compared between morphs using mixed-effects models constructed in SPSS 15.0 (SPSS Inc.) or SAS (PROC MIXED, SAS 9.1.2, SAS Institute 1994.) Models were constructed using a backward elimination strategy and individual was entered as a random effect.

Our analysis also considered the effects of phenology. Date was converted to “corrected day”: the julian date on which the sample was taken corrected for the date that the first egg of that year was found in the study site. Using the “corrected day” value allowed us to standardize our date variable between the three years of the study. This is advantageous because in 2004, breeding started approximately 9 days earlier than in either 2003 or 2005.

We performed a second analysis on the subset of birds whose nests we had located and monitored in order to examine the effect of stage in nesting cycle on differences in T between morphs. Due to sample size limitations we grouped birds that were sampled during pre-nesting, nest building and laying into a stage called “defense.” During the defense stage, birds may be either setting up territories, defending a fertile mate, or seeking extra-pair copulations activities

during which they exhibit aggressive behaviors. Females captured during incubation, nestling feeding and fledgling feeding were grouped into a stage called “parental.” In contrast, males captured during incubation were categorized as being in the defense stage. Males of this species do not incubate and have not been observed to feed incubating females, and are therefore not engaged in parental activities. Males were only classified as “parental” when they were feeding nestlings or fledglings. Males (n=46 individuals, 74 samples) and females (n=31 individuals, 38 samples) were analyzed separately. Testosterone levels were analyzed using a mixed-effects model with morph and stage as fixed factors and individual as a random effect.

Results of the laboratory GnRH challenge experiments were analyzed using repeated measures ANOVAs in the statistical software package, JMP 5.0.1. Data from the GnRH challenge performed in the field was analyzed using a two-tailed t-test (GraphPad Prism 4.00.)

Enzyme Immunoassay: White-throated Sparrow plasma testosterone levels were measured using an enzyme immunoassay kit from Assay Designs Inc. (catalog no. 900-065). These kits use raw plasma, which is added directly to the well. Steroid binding globulins, which may interfere with assay reactions, can be degraded by adding a steroid displacement buffer (SDB). Because these kits are designed to be used with a variety of biological fluids, plasma dilution and concentration of SDB must be optimized. Optimization for T was performed in a

similar manner to the optimization for corticosterone, as detailed in Wada et al. (Wada et al., 2007). Briefly, A sample of pooled white-throated plasma was stripped of endogenous testosterone by incubating plasma with a charcoal solution (1% norit A Charcoal and 0.1% dextran in assay buffer). This stripped plasma was then spiked with a known concentration of testosterone (500 pg/mL). Spiked, stripped plasma was assayed at four different dilutions (1:5, 1:10, 1:20, 1:30, diluted with assay buffer) with three different concentrations of SDB (0%, 1%, 2%) and compared to a standard curve on the same plate. Hence, each sample *should* read at 500 pg/ml T, unless there is interference from the plasma. For testosterone, a plasma dilution of 1:20 with no SDB added removed the interference of plasma compounds in the assay.

Individual plasma samples were thawed, picofuged, vortexed and diluted with assay buffer to a 1:20 concentration. Samples were aliquotted into separate wells in triplicate. The six-point standard curve (2,000 pg/mL to 8.2 pg/mL) and a separate external standard were also run in triplicate on each plate. Enzyme-labeled testosterone and antibody were added and the plate was incubated at 26°C on a shaker for 2 hours. Wells were then emptied and rinsed with wash buffer and enzyme substrate was added. The plate was incubated for one hour, again at 26°C, but without shaking. Stop solution was added after this final incubation and the plate was immediately read using a Multiskan Ascent microplate reader at 405 nm corrected at 595 nm. The lower limit of detectability for these assays was 1.6 pg per well, and all non-detectable samples were

assigned this value. External standards were used to calculate inter-plate variability (11.6 %), and intra-assay coefficients of variation were calculated using sample replicates (9.4 %).

## Results

### Testosterone

Effect of corrected day: *Females:* We found no significant difference in T between WS and TS females sampled in 2003 and 2004 . The linear mixed-effect model (Table 1) revealed no significant effect of the fixed factor morph ( $p=0.44$ ). Testosterone declined with day in both morphs, however this trend was not significant ( $p=0.07$ .)

*Males:* The linear mixed-effects model revealed a significant effect of the fixed factors morph ( $p=0.02$ ) and date ( $p=0.004$ ; Table 2). White-striped males had higher T than TS males and levels declined over the season in both morphs.

Effect of stage in nesting cycle: We found no interaction between morph and stage in either males or females. There was a significant effect of morph in males, with WS males having higher T levels than TS males in both stages ( $p=0.023$ ) (Table 4 and Fig. 1). Males tended to have higher T in the defense stage, though this effect was not significant ( $p=0.12$ ; Table 4.) There was no effect of morph or stage in females (Table 3 and Fig. 2).



Playback: Social interactions may increase plasma T in some species (Wingfield and Hahn, 1994; Wingfield et al., 1990a). As such, longer song-playbacks (a simulated social interaction) may influence T level. However, our use of playback to capture birds did not increase T in male WTSP (length of playback defined as time from start of playback to capture in the net). A linear regression revealed a loose but significant *negative* relationship between length of playback and plasma T levels (adjusted  $r^2 = 0.06$ ,  $p=0.04$ ,  $b = -0.13$ .) The negative slope of the correlation suggests that birds with higher T levels to begin with may have responded to playback and been captured more quickly than those with lower T; i.e., that T is influencing time to capture, not that length of playback is influencing T within our sampling timeframe. This effect should not bias our results because there was no difference in the length of playback needed to capture birds of the two morphs ( $N=51$ ,  $F = 1.18$ ,  $p=0.3$ ).

#### GnRH Challenge

Lab: Both males ( $n = 21$ ; 14 WS, 7 TS) (Fig. 3) and females ( $n = 10$ ; 5 WS, 5 TS) (Fig. 4) showed elevated T in response to GnRH injection compared to those injected with saline ( $p < 0.001$  and  $p = 0.003$  respectively). However there was no difference between morphs in either sex (males,  $p=0.99$ ; females,  $p=0.48$ ).

Field: We tested response to GnRH in free-living, breeding male WTSP (n=13; 7 WS, 6 TS) to ensure that the lack of morph difference observed in the lab was not due to a lack of environmental cues. As in the lab, we found no difference between morphs in response to GnRH injection (Fig. 5  $p=0.4$ ,  $t=0.88$ ,  $df=11$ ).

## Discussion

### Testosterone

Contrary to our prediction, we found no difference in T between WS and TS females. Thus T does not appear to mediate the difference in aggression between female morphs. Mechanisms of female aggression are poorly understood and many studies have found no relationship between T and aggression in females (Elekonich and Wingfield, 2000; Goymann and Wingfield, 2004). However, in the dunnock (*Prunella modularis*), females involved in repeated aggressive interactions in competition for mates exhibited higher testosterone levels (Langmore, Cockrem & Candy, 2002). It is possible that differences in aggression between WS and TS females may result from differences in other hormones such as estrogen or progesterone, as suggested by work in reptiles and mammals (Woodley, Matt and Moore, 2000; Woodley and Moore 1999 (*Sceloporus jarrovi*); Kapusta, 1998 (*Clethrionomys glareolus*)). Or aggression may even be influenced by the ratio between two hormones such as

testosterone and progesterone as (e.g. in the mouse, *Peromyscus californicus*; (Davis and Marler, 2003).)

This study demonstrates a significant difference in plasma T levels between WS and TS males and shows that this difference persists across both the defense and parental stages of the nesting cycle. These differences in plasma T are consistent with morph-specific differences in aggression, suggesting that T may mediate this difference in male behavior. It is noteworthy that WS males also had higher T during the parental stage. Given the effects of T on parental behavior in other species (Schoech et al., 1998; Schwagmeyer et al., 2005; Van Roo, 2004), it is possible that T may also influence differences in parental behavior between male morphs.

It must be emphasized that the magnitude of the difference in plasma T between morphs is small. In all analyses of male morphs, models indicated that the difference between morphs was between 0.5 and 0.6 ng/mL. The biological relevance of such a small amount of T is unclear, as there have been very few studies evaluating the dose-response effect of T on behavior. One factor that may be reducing the magnitude of the difference that we detect is individual variation. Variation between individuals, and thus within morphs, is to be expected because an individual's "testosterone phenotype" results from an interaction between its genotype and the environment. In this species, there is a demonstrated genetic component to the phenotype (morph-type is determined by

a peri-centric chromosomal inversion (Thornycroft, 1966).) However, factors in both the developmental and immediate social environment may modify the testosterone phenotype (Wingfield 1985). These factors are not expected to vary systematically with morph type (but see (Formica et al., 2004)) and therefore will add noise to any morph-specific pattern in testosterone.

Despite potential individual differences in developmental history and social environment, we still detect a consistent difference between male morphs. In a similar study, Spinney et al. (Spinney et al., 2006) also found similar (small) differences in T between free-living male morphs captured in May. Taken together our results suggest that differential regulation of T may play a part in mediating differences in behavior between male morphs. We also suspect that other hormonal or neural systems are playing an important role. For example, Maney et al (2005) recently demonstrated that the WS morph has more vasotocin innervation in brain areas associated with agonistic behavior. Vasotocin is a neuropeptide hormone that has been associated with aggressive behavior in some species (Goodson et al., 2004; Maney et al., 1997).

The difference in T between WS and TS males is consistent with results in other behaviorally polymorphic species, in which the more aggressive morph has higher levels of androgens. In side-blotched lizards, orange territorial males have significantly higher levels of T than do the less aggressive blue males, or yellow sneaker males (Sinervo et al., 2000). Similarly in the midshipman fish, territorial

males have higher levels of 11-ketotestosterone than do sneaker morphs (Oliveira et al., 2002). In contrast, previous studies of the white-throated sparrow failed to show a difference in testosterone levels between WS and TS individuals (DeVoogd et al., 1995; Schlinger, 1987; Schwabl et al., 1988). Two of these studies were conducted using non-breeding, captive birds in which we would expect T levels to be low and therefore differences would be difficult to detect. DeVoogd et al (1995) measured plasma T during the breeding season when it should have been elevated and found no differences between morphs. However, given the small effect sizes and large inter-individual variation, it is not surprising that other studies did not detect a difference between morphs.

### GnRH Challenge

Male morphs differ in plasma T levels, but what is the physiological difference between the morphs that leads to this difference in T? The GnRH challenge experiment helps us localize the mechanism that leads to differences in T secretion. In this experiment, the pituitary is stimulated with an exogenous dose of GnRH, thus activating the pituitary-gonadal axis to secrete T. Because individuals are given equal doses of GnRH, any differences in T secretion can be interpreted as differences in the ability of either the pituitary or gonad to respond to the secretagogue.

We found no difference between morphs in response to GnRH injection. The lack of difference in this study suggests the morph-difference in plasma T stems from a difference in testosterone regulation at the hypothalamus or higher brain regions, rather than a difference in the pituitary or gonad. This is consistent with Spinney et al (2006) who found no differences between morphs in LH levels following GnRH injection. Interestingly, their study did report a greater elevation of T in response to GnRH in WS males. Thus, it is not entirely clear at which level in the hypothalamic-pituitary-gonadal axis differences in T are generated. Potential factors at the level of the hypothalamus include differences in the number of GnRH secreting neurons, or the number of testosterone receptors involved in negative feedback.

This study demonstrates a difference in plasma testosterone that correlates with the observed differences in aggression between male white-throated sparrow morphs, and shows that this difference persists during both the aggressive and parental phases of the nesting cycle. Furthermore, it suggests that the mechanism responsible for this difference in plasma T may lie at or above the hypothalamus. It remains to be seen what mechanisms, hormonal or otherwise, underlie differences in aggression between female morphs. Our findings comprise an essential first step in the investigation of the endocrine mechanisms responsible for generating a behavioral polymorphism in the white-throated sparrow. This species is an interesting case study in and of itself, but it is our

hope that it will also provide a useful model system in which to compare and contrast distinct behavioral phenotypes within populations.

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**Table 1. Female Testosterone in Relation to Morph and Corrected Day**  
Linear Mixed-Effects Model

Fixed Effects	Standard Estimate	Error	F	df	P-value
Intercept	0.3105	0.06048	-	-	-
Morph	0.05109	0.06408	0.64	11	0.44
Corrected day	-0.00243	0.001191	4.15	11	0.066

**Table 2. Male Testosterone in Relation to Morph and Corrected Day**

Linear mixed-effects model

Fixed Effect	Standard Estimate	Error	F	df	P-value
Intercept	0.874	0.056	-	-	-
Morph	-0.202	5.705	5.88	40	0.019
Corrected Day	-0.013	9.065	9.46	40	0.003

**Table 3. Female Testosterone in Relation to Morph and Nesting Stage**

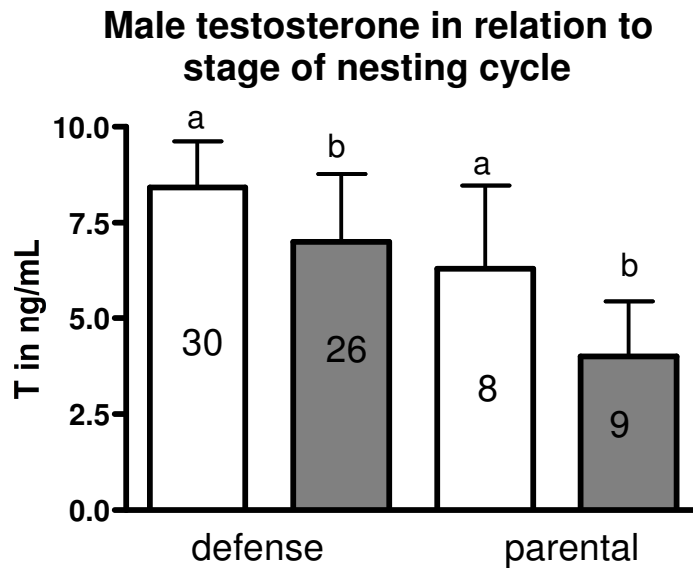
Mixed Repeated Measures Model-Females

Fixed Effects	Numerator df	Denominator df	F	P-value
Morph	1	27	0.44	0.51
Stage	1	1	5.25	0.262

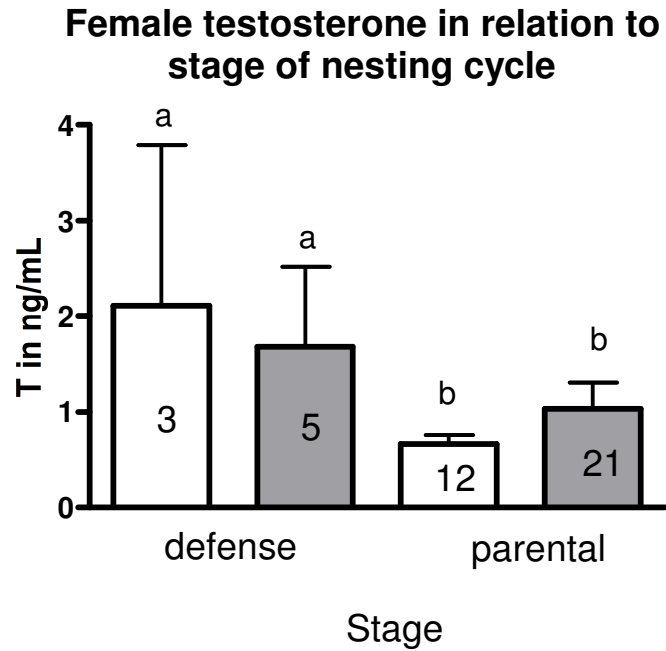
**Table 4. Male Testosterone in Relation to Morph and Nesting Stage**

Mixed Model- Males

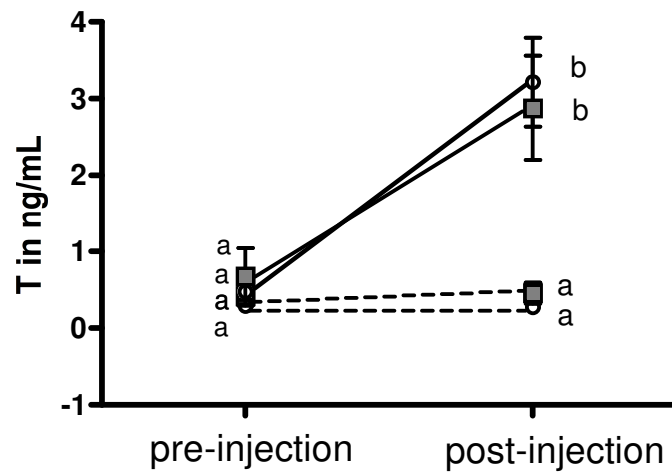
Fixed Effect	Numerator df	Denominator df	F	P-value
Morph	1	37	5.622	0.023
Stage	1	68	2.495	0.119



**Figure 1:** Plasma testosterone in males during defense and parental stages of nesting cycle. Gray bars represent mean T in TS males, White bars represent mean T in WS males. Error bars represent SEM. Sample sizes are indicated inside the bars. Bars that do not share a lettered superscript are significantly different according to the linear mixed-effects model. T tends to be higher during the defense stage in both morphs. WS males had significantly higher T than TS males in both the defense and parental stages.

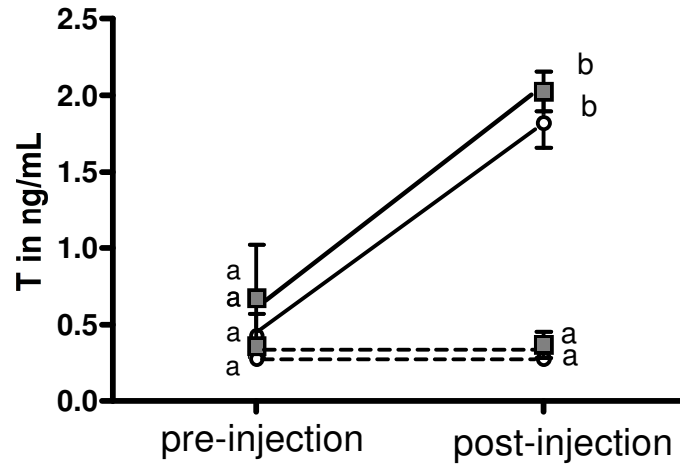


**Figure 2:** Plasma testosterone in females during defense and parental stages of nesting cycle. Gray bars represent mean T in TS females, White bars represent mean T in WS females. Error bars represent SEM. Sample sizes are indicated inside the bars. Bars that do not share a lettered superscript are significantly different according to the linear mixed-effects model. T is significantly higher during the defense stage in both morphs, however there was no significant difference between morphs.

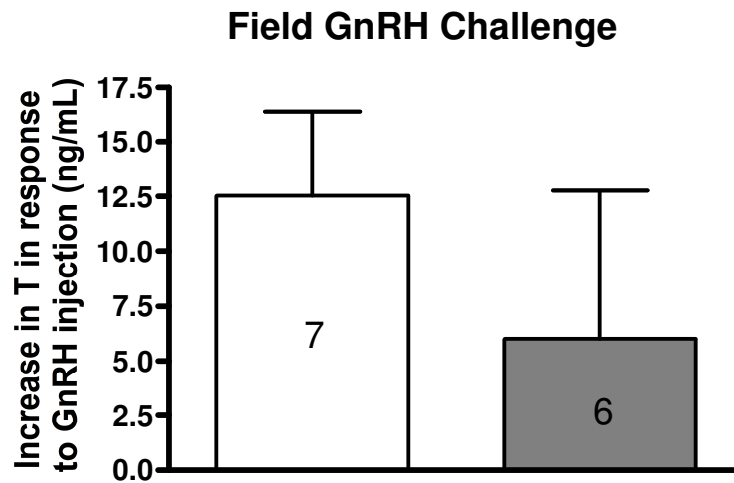


**Figure 3:** Response of captive WS (gray squares,  $n = 14$ ) and TS (open circles,  $n = 7$ ) males to GnRH (solid lines) or saline (dashed lines) injections. Data are plotted as mean  $\pm$  SEM. GnRH significantly increases T levels, but does so equally in both morphs.

### GnRH Challenge in Captive Females



**Figure 4:** Response of captive WS (gray squares,  $n = 5$ ) and TS (white circles,  $n = 5$ ) females to GnRH (solid lines) or saline (dashed lines) injections. Data are plotted as mean  $\pm$  SEM. GnRH significantly increases T levels, but does so equally in both morphs.



**Figure 5:** Increase in plasma T in free-living males injected with exogenous GnRH. Bars indicate mean + SEM and sample size is indicated within bar. There was no significant difference between morphs (white bar= WS males; tan bar=TS males.) Power analysis indicates that a sample size of 92 individuals per morph would be needed to detect a difference in response between morphs with  $\beta = .10$ .



## Chapter 2

### **INTERACTION OF TESTOSTERONE, CORTICOSTERONE AND CORTICOSTERONE BINDING GLOBULIN IN THE WHITE-THROATED SPARROW (*ZONOTRICHIA ALBICOLLIS*)**

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## Abstract

According to the “Free Hormone Hypothesis”, hormone molecules bound to a binding globulin protein cannot cross membranes and are restricted from entering tissues. Only unbound, “free” hormone, which enters tissues, is biologically active. Mammals have both sex hormone binding globulin (SHBG, binding both testosterone and estrogen) and corticosteroid binding globulin (CBG, binding primarily glucocorticoids and progesterone). Birds, in contrast, have no detectable SHBG, leading to the early conclusion that birds have no plasma regulation of testosterone or estrogen. CBG, however, can bind androgens with relatively high affinity. In birds, therefore, the control of androgenic effects may be tightly regulated by glucocorticoid physiology because glucocorticoids compete with androgens for CBG binding sites. In order to fully understand testosterone physiology, one must determine what portion of T is bound to CBG and what portion is free (unbound). Here we report plasma levels of total T, total CORT, and CBG in both captive and free-living White-throated Sparrows. From these data we then calculate levels of free T. The White-throated Sparrow is a behaviorally polymorphic species in which morphs differ in their expression of territorial aggression. A comprehensive evaluation of T, CORT and CBG interactions is essential to understanding the potential hormonal mechanisms of morph behavioral phenotypes. We found no significant differences in levels of CORT or CBG between the morphs or sexes. While CBG has a higher binding affinity for CORT than for T, we estimated approximately

96% of T was bound to CBG. In free-living birds we found that the more aggressive white-striped males had higher levels of total T than did tan-striped males, however no difference existed between female morphs. In captivity, we saw no morph-specific differences in total T in either males or females (although trends matched patterns in free-living animals). Despite the substantial binding between T and CBG, the lack of significant morph or sex-specific differences in CORT or CBG capacity cause patterns of free T to mirror those of total T. While CBG has the potential to greatly influence T availability to tissues, in this species the interaction of T, CBG and CORT does not appear to alter general patterns of T availability.

### Introduction

Binding globulins are glycoproteins that bind steroid hormones in the plasma with high affinity. According to the free hormone hypothesis, hormone molecules bound to binding globulins are unable to enter tissues and thus are considered biologically inactive (Mendel, 1989). “Free” or unbound hormone is therefore thought to be the biologically relevant portion of the total amount of hormone in the plasma; thus, binding globulins appear to be a significant regulator of hormone activity at the plasma level. Mammalian and biomedical endocrine studies commonly distinguish between total and free hormone (D'Agostino et al., 1982; Leeper et al., 1988; McDonald and Taitt, 1982; Osuna et al., 2006; Perret,

1986; Yeap et al., 2007). And while the majority of endocrine studies in non-model organisms simply measure total hormone, an increasing number of comparative studies are considering binding globulins (Landys et al., 2007; Love et al., 2004; Lynn et al., 2007; Zysling et al., 2006). Several of these studies suggest a significant role for binding globulins in mediating the effects of steroid hormones (Jennings et al., 2000; Lynn et al., 2003; Wada et al., 2007).

Mammals have separate binding globulins for sex steroids and glucocorticoids. Sex hormone binding globulin (SHBG) binds testosterone and estradiol while corticosteroid binding globulin (CBG) binds cortisol and corticosterone. Birds, however, lack SHBG; and until recently, physiologists believed that avian sex steroids were not regulated by binding globulins at the plasma level. However more recent studies show that in birds, CBG binds androgens with high affinity. In several avian species, CBG binds testosterone (T) with 10-fold lower affinity than glucocorticoids (Deviche et al., 2001; Wingfield et al., 1984); in spite of this decreased affinity, CBG apparently binds >90% of circulating T; this study).

Hence, in birds, both glucocorticoids and testosterone compete for CBG. This presents the possibility for an intriguing interaction between the reproductive and stress-reactive systems at the plasma level. As glucocorticoid levels change (due to either diel cycles or stress-related events), the amount of CBG available to bind T will change. For example, as plasma CORT increases up to 10-fold with stress, T will be displaced from CBG; this could result in a spike of free T

available to tissues, as well as a more rapid clearance of the T present.

Alternatively, effects could be seen at non-stress levels; if sexes or morphs have varied levels of CBG or CORT, then more or less T could be bound in the plasma. Thus the actions and metabolism of T will be affected by both CBG and CORT and a thorough examination of T physiology must consider these interactions.

Researchers interested in the effects of testosterone on behavior often look to behaviorally polymorphic species. These species are of particular interest when studying hormonal mechanisms of behavior because they allow researchers to make more powerful comparisons. While most behavioral phenotypes are continuously distributed, behaviorally polymorphic species present a bimodal distribution of phenotypes. Typically when researchers want to compare distinct behavioral phenotypes, they must compare individuals of different species, unavoidably introducing confounding factors such as differences in ecology or phylogeny. By using polymorphic species, researchers can find substantial differences in behavior between individuals of the same species.

The goal of this study is to determine the influences of both CBG and CORT on testosterone physiology in the White-throated sparrow. This species exhibits a unique behavioral polymorphism that is manifested in both sexes. Morphs differ in both aggressive and parental behavior. White-striped morphs respond more aggressively to simulated territorial intrusion (Kopachena and Falls, 1993a) and

tan-striped morphs provision nestlings at a higher rate (Kopachena and Falls, 1993b). Testosterone has effects on both territorial aggression and nestling provisioning behavior in many temperate species of birds (Schwagmeyer et al., 2005; Van Roo, 2004). Previous studies (Spinney et al., 2006; Swett and Breuner, 2004) Swett and Breuner, submitted) have shown a difference in total T between male morphs, but the effects of CBG and CORT on testosterone physiology have not previously been considered.

## Methods

### Study Species

The white-throated sparrow (WTSP) is a migratory songbird that breeds in the northern U.S. and Canada and winters primarily in the southeastern U.S. (Falls and Kopachena, 1994). Breeding sparrows were observed and captured on forested property owned or managed by the Northwoods Stewardship Center in East Charleston, Orleans County, Vermont. Samples were collected between 22 May and 29 July 2003, 2 May and 23 July 2004 and 21 April and 10 July 2005. Sparrows were captured in seed-baited Potter traps or mist-nets. Blood samples were collected by venipuncture of the alar vein and blood was drawn into a heparinized microhematocrit tube via capillary action. Blood used to measure baseline CORT was collected within 3 minutes of the bird contacting the net or the fieldworker approaching the potter trap. Samples used to measure T and

CBG were collected within 10 minutes of the onset of capture stress so as to minimize the effect of stress on T levels.

Blood samples were kept on ice while in the field (up to four hours.) As soon as possible the sample was centrifuged and plasma drawn off using a Hamilton syringe. Plasma was kept frozen at approximately -20°C until it could be assayed.

Lab birds: Wintering sparrows were captured in Travis County, Texas in seed-baited Potter traps or seed-baited mist nets. Birds were housed in individual cages 33 cm x 38 cm x 43 cm in captivity and were subjected to a long-day (14 light:10 dark) photoperiod for 3 weeks prior to the start of sampling. This photoperiod induced birds to come into a pseudo-breeding condition indicated by an increase in singing. All blood samples used to measure CORT were collected within 3 minutes of the experimenters entering the room. Samples used to measure T and CBG were collected within 10 minutes.

#### Enzyme Immunoassay

White-throated Sparrow plasma testosterone levels were measured using an enzyme immunoassay kit from Assay Designs (cat # 901-065). These kits are designed to use raw plasma, which is added directly to the well. A steroid

displacement buffer (SDB) can be used to degrade steroid binding globulins that may interfere with the assay. Plasma dilution and concentration of SDB must be optimized prior to the measurement of samples. A sample of pooled white-throated plasma was stripped of endogenous testosterone by incubating plasma with a charcoal solution (1% norit A Charcoal and 0.1% dextran in assay buffer). This stripped plasma was then spiked with a known concentration of testosterone (500 pg/mL). Spiked, stripped plasma was assayed at four different dilutions (1:5, 1:10, 1:20, 1:30) with three different concentrations of SDB (0%, 1%, 2%) and compared to a standard curve on the same plate. A plasma dilution of 1:20 with no SDB added minimized the interference of plasma compounds in the assay.

Individual plasma samples were thawed, picofuged, vortexed and diluted with assay buffer to a 1:20 concentration. Samples were aliquotted into separate wells (100 $\mu$ L per well, each in triplicate.) The standard curve, including 6 standards ranging from 2,000 pg/mL to 8.2 pg/mL, and a separate external standard were also run in triplicate on each plate. Conjugate and antibody were added and the plate was incubated at 26°C on a shaker for 2 hours. Wells were then emptied and rinsed with wash buffer and a second substrate was added. The plate was incubated for one hour, again at 26°C but without shaking. Following the second incubation, stop solution was added to each well and the plate was immediately read using a Multiskan Ascent microplate reader at 405 nm corrected at 595 nm. The lower limit of detectability ranged from 0.8 to 3



pg/well. Morphs and sexes were randomly distributed across assays and across plates within an assay.

Plasma CORT was also measured using an Assay Designs corticosterone enzyme immunoassay kit (cat # 901-097) following a similar protocol optimized for use in the congeneric White-crowned sparrow and checked for accuracy in this species (for detailed description of protocol and optimization see Wada et al, 2007). Plasma was diluted 1:40 with assay buffer and a 1% concentration of SDB solution was used. The lower limit of detectability in this assay ranged between 1.58 pg/well and 3 pg/well. Again, individuals were distributed randomly across and within assays.

#### Corticosterone Binding Globulin characterization and assay.

Plasma Preparation: Plasma was stripped of endogenous CORT via incubation with dextran-coated charcoal solution (0.1% dextran, 1% norit A charcoal in 50mM Tris) for 30 minutes at room temperature. Samples were centrifuged and supernatant removed. Plasma samples were assayed at a final dilution of 1:1089.

Equilibrium Saturation Binding Analysis: An equilibrium saturation binding analysis was performed using pooled plasma. Plasma was incubated at 4 °C for 2 hours with different concentrations of [ $H^3$ ] CORT ranging from 0.23 nM to 12 nM in the presence or absence of unlabelled CORT. Immediately following incubation, plasma solutions were passed via rapid vacuum filtration over PEI (0.3%) soaked Whatman GF/B paper filters. Filters were rinsed three times with 3 mL ice cold 25nM Tris.

Radioactivity bound to filters was quantified via standard liquid scintillation spectroscopy. Briefly, filters were allowed to dry, placed in scintillation vials, 100  $\mu$ L isopropyl alcohol and 5 mL scintillation fluid were added (Scint Safe 50%, Fisher or Perkin Elmer Ultima Gold.) Radioactivity was measured in a Beckman-Coulter LS 6500 Multi-purpose Scintillation counter or a Perkin Elmer TriCarb 2900TR scintillation counter.

Relative affinity of CBG for Testosterone and Corticosterone: The relative affinity of CBG for corticosterone versus testosterone was determined with a competition curve experiment in which radiolabeled CORT competes with either unlabeled CORT or unlabeled testosterone for CBG binding sites. Briefly, plasma was stripped as described above and incubated for 2 hours on ice with 2nM [ $H^3$ ] CORT and unlabeled competitor (either CORT or T) ranging in dilution from 1  $\mu$ M to 0.1 nM.

Point Sample Analysis: CBG in all field and lab samples was assayed with a point sample analysis at 4 °C. Morphs and sexes were distributed randomly across assays and across filters within an assay. Plasma samples were prepared as described above. Samples were then incubated on ice for 2 hours with 20 nM [ $H^3$ ] CORT and 1  $\mu$ M unlabeled CORT as a competitor. This concentration of radio-labeled ligand was estimated by the equilibrium saturation binding analysis to occupy approximately 91% of binding sites. Radioactivity bound to filters was quantified via liquid scintillation spectroscopy as described above. Inter-assay variability was 17.5% (field samples).

## Results

### CBG characterization

The equilibrium saturation binding analysis (Fig. 1) indicated that white-throated sparrow CBG binds CORT with high affinity ( $EC_{50}$ = 4.17 nM). The Scatchard-Rosenthal replot of the data (Fig.1 inset) demonstrates that there is a single binding site for CORT on CBG. A competition curve experiment (Fig. 2) shows that while CBG binds CORT with higher affinity it also binds T with relatively high affinity ( $EC_{50}$ = 28.18 nM).

### CBG Binding Capacity

Among the captive birds, we found no significant differences in CBG binding capacity between morphs or sexes (ANOVA  $p=0.08$ ; Fig. 3a). However females tended to have a higher binding capacity. This trend was reversed in the free-living sparrows with males tending to have higher CBG binding capacity, though there were no significant differences between morphs or sexes (ANOVA  $p=0.055$ ; Fig 3b).

#### Baseline Corticosterone

We found no significant differences between morphs or sexes in total plasma corticosterone in either captive (ANOVA  $p=0.46$ ; Fig. 4a) or free-living (ANOVA  $p=0.3$ ; Fig. 4b) sparrows. Total Plasma CORT tended to be higher in free-living birds (ANOVA  $p=0.0002$ ).

#### Total Testosterone

Total plasma testosterone tended to be higher in captive WS males compared to captive TS males, however this difference was not significant (ANOVA  $p=0.34$ ; Fig. 5a). Among free-living sparrows this difference between male morphs was robust (ANOVA  $p<0.0001$ ; Tukey-Kramer HSD  $q=2.66$ ,  $p<0.05$ ; Fig. 5b).

Surprisingly, total plasma T levels in TS males were similar to those of TS females. Overall, T was significantly higher in free-living birds than in captive birds (ANOVA  $p<0.0001$ .)

### Free Testosterone

Patterns of free T mirrored those of total T with WS males having significantly higher levels than TS males or females in the free-living populations (ANOVA  $p=0.0085$ , Tukey-Kramer HSD,  $q=2.66$ ,  $p<0.05$ ; Fig. 6B). A similar pattern was seen in the captive birds, but it was not significant (ANOVA  $p=0.61$ ; Fig. 6a).

### Power Analyses

Individual variation in hormone and CBG measures was high. Post-hoc power analyses ( $\beta=0.10$  and  $p=0.05$ ) indicated that we would need to sample 17 individuals of each morph-sex group in order to detect a significant difference in total T among captive birds. Detecting differences in CBG capacity would require samples of 15 individuals (lab) or 21 (free-living) per morph-sex group. Total CORT was highly variable and a sample of 42 (lab) or 80 (free-living) individuals per morph-sex group would be required to detect a significant difference. Samples sizes this large are not usually feasible in a study of this type. Because it is derived from the other measures, our power to detect differences in free-T is limited by the variability of total CORT.

## Discussion

Binding between CBG and T has been calculated in three species of passerines, and in each it is estimated that over 90% of testosterone is bound to CBG (96% this study, 93.6% Deviche et al, 2001, 98% calculated from Lynn et al, 2007). Thus, CBG has the potential to significantly modify the actions of testosterone once it has been secreted. Differences in CBG binding capacity between two individuals with identical levels of total testosterone could lead to significant differences in free testosterone, which may be the most biologically relevant fraction.

In this study, we found that free-living male morphs differed in total testosterone levels such that more aggressive WS morph had higher total T than did the TS morph. This is in agreement with our previous findings and those of Spinney et al (2006). However, while free-living WS males had significantly higher levels of CBG than did females, they were not significantly higher than those of TS males. Neither CBG nor CORT differed substantially between the morphs or sexes. Therefore, morph specific patterns in free-T resembled those of total T. Thus, we conclude that the interactions between CBG, T and CORT probably do not contribute to the observed differences in aggression between the morphs.

To our knowledge, only one other study has examined the role of binding globulins in a behaviorally polymorphic species. In 2000, Jennings et al.

documented a difference in androgen-glucocorticoid binding globulin (AGBG) binding capacity between territorial and non-territorial morphs of the tree lizard (*Urosaurus ornatus*). Like CBG, AGBG binds both testosterone and corticosterone. They hypothesized that this lower binding capacity in non-territorial males leads to a greater increase in free CORT during stress events in these males compared to territorial males. This greater free CORT could have an inhibitory effect on T, and lead to the stress induced reduction in T in non-territorial morphs observed by Knapp and Moore (Knapp and Moore, 1997)

Our study considered only baseline CORT levels. Stress induces rapid increases in plasma CORT levels. The increase in CORT during acute stress could lead to transient increases in free T as rising CORT titers push more T off of CBG. For instance, Lynn et al. suggested that the decreases in T following territorial intrusion observed by Van Duyse et al (Van Duyse et al., 2004) could have been due to the fact that stress of the encounter lead to an increase in CORT, which in turn lead to an increase in the proportion of T that was free. Free T would be available for clearance by the liver and hence total plasma T would decrease. If WTSP morphs differ in the amount of CORT they secrete in response to acute stress, this could lead to transient differences in free T levels. Our preliminary evaluation of the stress response in photo-stimulated, captive WTSP found no difference in stressed levels of CORT between morphs. However, Schwabl (1995) found that photo-stimulated, captive WS males secreted greater amounts of CORT in response to stress (capture and 60

minutes of restraint) than did TS males. This pattern was reversed in males held on short-day photoperiods, when TS males secreted more CORT.

Many possible factors could also affect free T levels; one of these is progesterone. Avian CBG binds the sex steroid progesterone ( $P_4$ ) with equal or greater affinity than corticosterone. Thus  $P_4$  will also compete with T for binding sites on CBG. Patterns of  $P_4$  secretion during the breeding season vary between bird species (Dawson, 1983; Heath et al., 2003; Schoech et al., 1991) and have not been evaluated in white-throated sparrows. If WS and TS morphs differ in their levels of plasma progesterone, such a difference could lead to a difference in availability of CBG and hence free testosterone. However,  $P_4$  levels are often quite low (~15 nM) in breeding birds, and CBG capacity varies between 100 and 300nM in this study. Hence, the impact is most likely small.

CBG is a relevant modifier of T action in the white-throated sparrow. However, morphs do not differ in either CBG levels or baseline CORT levels and so morph-specific patterns of free-T mirror patterns of total T. This study represents one of a growing number of studies in the comparative literature that addresses the role of binding globulins in regulating steroid action. While debate remains as to whether free steroid, rather than total or bound hormone, is truly the most biologically relevant measure, it is clear that binding globulins will affect the route of steroid uptake by cells. A thorough understanding of steroid action requires that the actions of binding globulins be considered.



### Acknowledgements

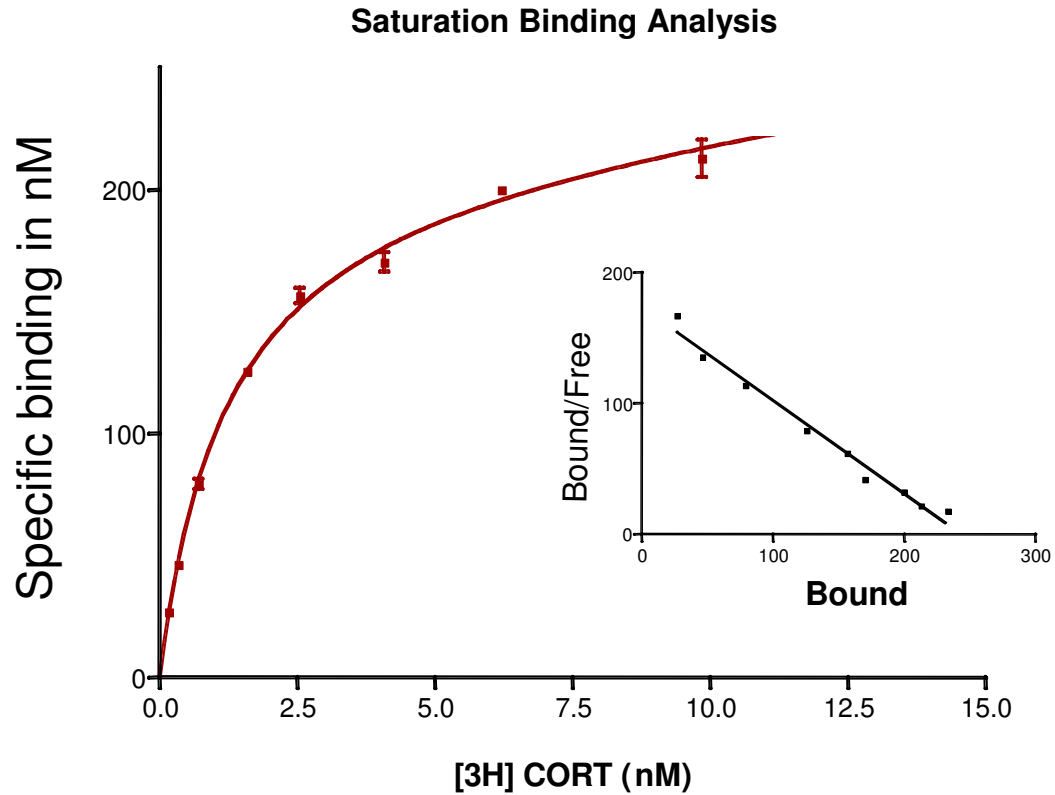
We thank Glennis Julian and Haruka Wada for assistance with the assays. Samantha Burrell and Ruby Dale-Brown provided able assistance in the field. We are indebted to the Northwoods Stewardship Center, The Center for Environmental Research at Hornsby Bend and The Brackenridge Field Laboratory at the University of Texas for allowing us access to field sites. M. Swett is especially grateful to the staff of the Northwoods Stewardship Center and the Stevens Family for support in VT. This work was funded by a NSF Graduate Research Fellowship to MBS, graduate fellowship support from the Ecology, Evolution and Behavior Graduate Program at the University of Texas at Austin, and NSF IBN-0236536 to CWB.

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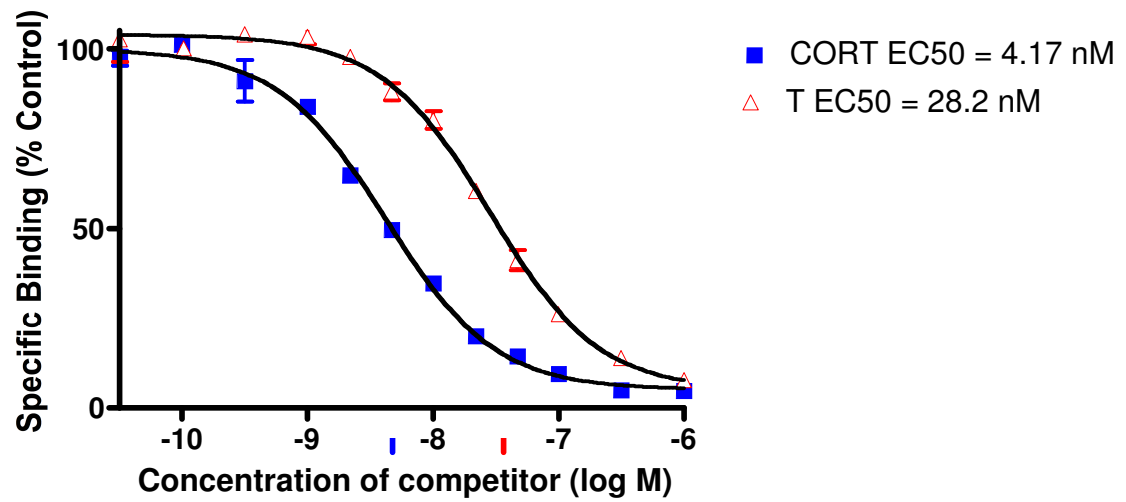
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**Figure 1.** Saturation binding curve and Scatchard-Rosenthal replot (inset) for 3H-Corticosterone binding to CBG in White-throated Sparrow Plasma. (A saturation binding curve must be done to characterize CBG affinity in each species. The Scatchard-Rosenthal replot is included to visualize whether or not the receptor (CBG) has one binding site or two. In this case, the linear relationship indicates that there is a single binding site.)

### Competition Binding Experiment



**Figure 2.** Competition binding of T and CORT to CBG in the White-throated Sparrow. CORT binds CBG with higher affinity (left arrow), however T (right arrow) also binds CBG with relatively high affinity.

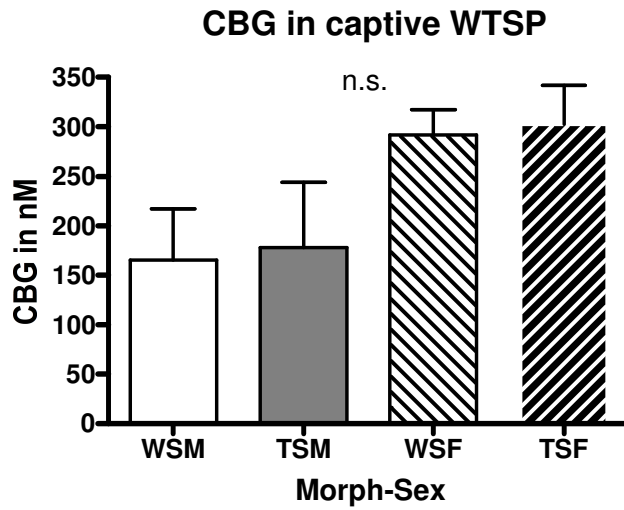


Fig. 3a

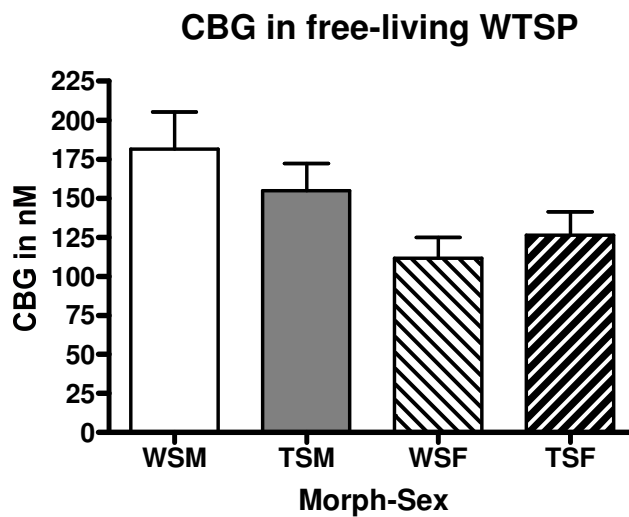


Fig. 3b

**Figure 3:** Bars indicate means + SEM. **a. Lab-** Though females tended to have higher levels of CBG, there were no substantial or significant differences between the morphs or sexes. (WS males= white bar, N=8; TS males= grey bar, N=9; WS females = striped white bar, N=6; TS females= striped black bar, N=5.) **b. Free-living-** In the field, the trend was reversed with males having higher levels of CBG, though only WS males were significantly higher than females. Sample sizes for free-living birds: WS males=14, TS males=13, WS females=10, TS females=15.

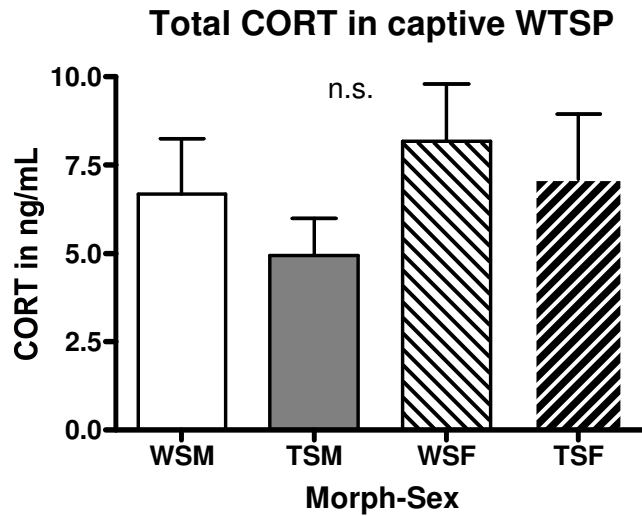


Fig. 4a

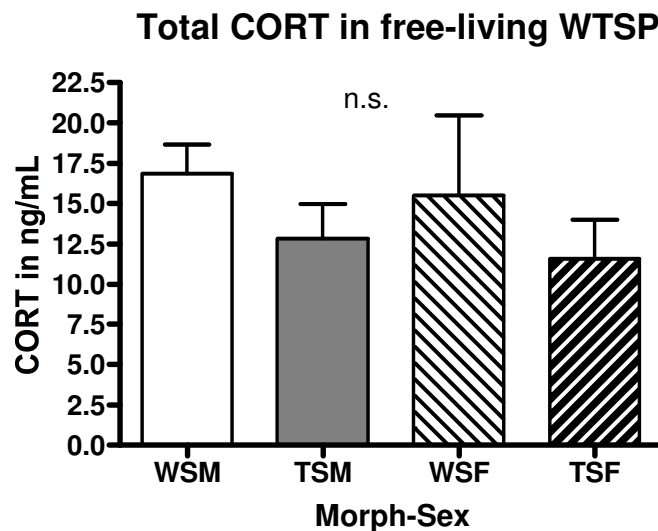


Fig. 4b

**Figure 4:** Bars indicate means + SEM. Bars that do not share a lettered superscript indicate that means are significantly different ( $p < 0.05$ ). **a-** Total plasma CORT in photostimulated, captive birds. There were no significant differences between morphs or sexes. (WS males= white bar,  $N=8$ ; TS males= grey bar,  $N=9$ ; WS females = striped white bar,  $N=6$ ; TS females= striped black bar,  $N=5$ .) **b-** As in captive birds, there were no significant differences in levels of total plasma CORT in free-living birds.

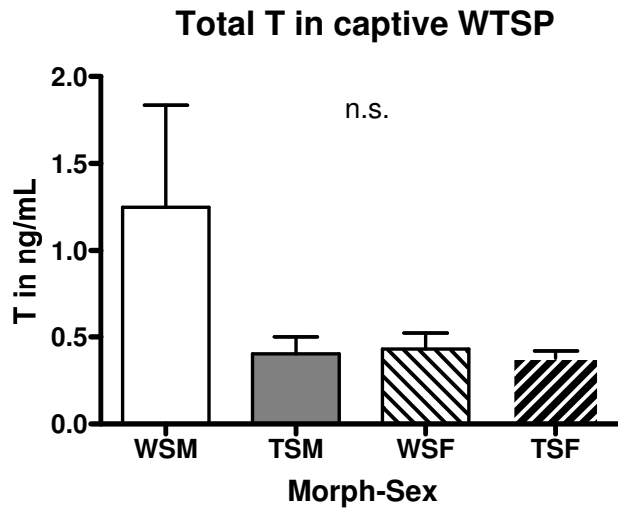


Fig. 5a

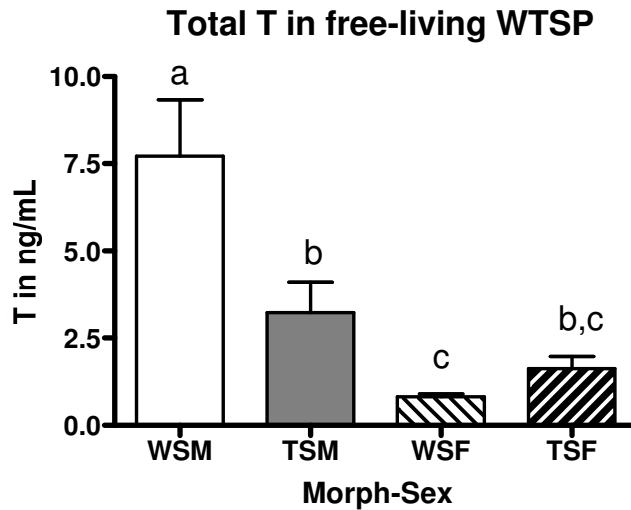


Fig. 5b

**Figure 5:** Bars indicate means + SEM. Bars that do not share a lettered superscript indicate that means are significantly different ( $p < 0.05$ ). **a-** Patterns of Total T in photo-stimulated birds kept in the lab were similar to those in the field. WS males tended to have higher total T however this difference was not significant. . (WS males= white bar, N=8; TS males= grey bar, N=9; WS females = striped white bar, N=6; TS females= striped black bar, N=5.) **b-** Total plasma T levels in free-living White-throated Sparrows. WS males had significantly higher testosterone than TS males, WS females and TS females.



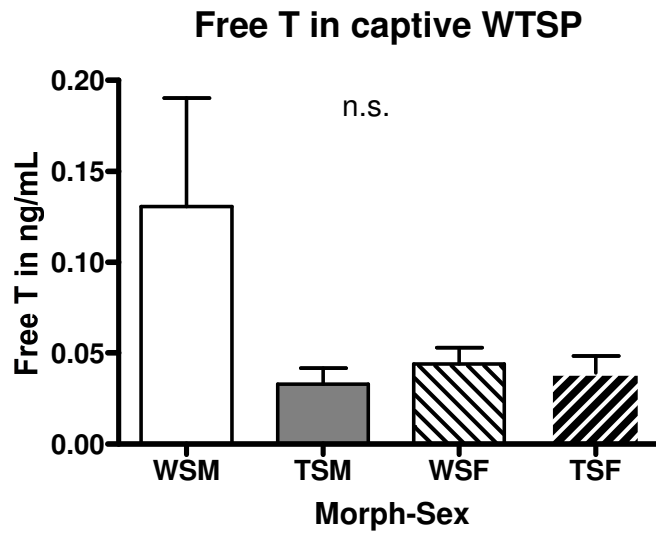


Fig. 6a

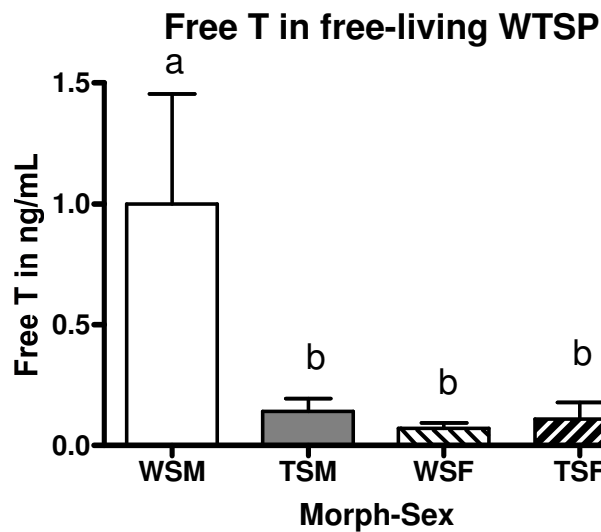


Fig. 6b

**Figure 6:** Bars indicate means + SEM. Bars that do not share a lettered superscript indicate that means are significantly different ( $p < 0.05$ ). In both captive (**a.**) and free-living birds (**b.**), patterns of free T mirrored those of total T because there were no significant or substantial differences in either CORT or CBG between the morphs or sexes. (WS males= white bar; TS males= grey bar; WS females = striped white bar; TS females= striped black bar.) (Sample sizes as in Fig. 5.)

## **Chapter 3**

### **Effect of Social Interaction on Levels of Total Plasma T and “Free” Plasma T in the White-throated Sparrow**

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## Abstract

Social instability is known to affect levels of circulating steroids. In many studies, androgen levels change during the unstable period but return to prior levels once hierarchies have been established. A recent study in *Anolis* lizards, however, indicates that *free* testosterone levels remain elevated in dominant males for an extended period of time. Here we examined behavioral and endocrine correlates of dominance relationships in captive male White-throated Sparrows, a polymorphic species in which morphs differ in their levels of aggression. Photo-stimulated males, one white-striped (WS) and one tan-striped (TS), were housed together sharing a single food dish. Blood samples were taken just prior to and 4 days following pairing for measurement of total plasma levels of testosterone (T), corticosterone (CORT) and corticosterone binding globulin (CBG) as well as estimates of free T (T that was not bound to CBG and therefore less available to tissues). We characterized the behavioral interactions involved in dominance establishment and determined which male became dominant. In this study, intense aggressive interactions (attacks and threats) were infrequent and only WS males engaged in these behaviors (except for a single TS male who displayed threat behavior). Displacements were the most common behavioral interaction and WS males displaced TS males significantly more frequently than vice versa. There was no difference between morphs in frequency of song. Using these and other measures to determine dominance, WS males became dominant in 6 of 8 pairs. While WS males tended to dominate TS males in this

study, this trend was not significant. Neither total, or free T was predictive of future social status, nor did we observe persistent changes in T, CORT or CBG after the social hierarchy was established. Contrary to the recent findings in *Anolis*, we found no difference in free T following hierarchy establishment. We conclude that WS males engage in more intense and frequent aggressive interactions than do TS males, however this difference is not reflected in their testosterone physiology either before or four days after pairing. In this study, the behavioral interactions involved in dominance establishment did not result in changes in either total or free T.

### Introduction

The social environment of an individual has profound effects on its hormone physiology. For instance, the presence of a mate or offspring can affect levels of sex steroids and prolactin respectively (Fleming et al., 2002; Silverin and Westin, 1995). One of the most common social interactions is a dominance relationship. Dominance relationships occur in a range of species from highly social animals to animals that are only temporarily gregarious, such as flocking winter birds. Even territoriality can be considered a form of dominance relationship in which status is determined by spatial location (Francis, 1988).

Many studies have investigated changes in hormones, especially glucocorticoids and androgens, associated with these dominance interactions. The establishment of a new dominance relationship is characterized by frequent agonistic interactions or “social instability”. The socially unstable period is often marked by elevations of androgens (typically testosterone) and glucocorticoid (GC) levels in one or both members of the dyad. (Blanchard et al., 2002; Bronson, 1973; Overli et al., 2004).

These acute elevations of testosterone (T) and GCs during establishment are transient, but individuals may show persistent changes in their T and GC physiology post-establishment. While T levels may be similar in dominants and subordinates once social instability has ended, in some species, continuing social stress can lead to decreases in T in subordinates (D.C. Blanchard et al 1993; Sachser and Prove 1986). Similarly, relative levels of GCs between dominants and subordinates depend on the costs of attaining and maintaining a given social status (Goymann and Wingfield, 2004). If dominant animals are frequently challenged by subordinates, dominants may exhibit higher levels of GCs. In contrast, subordinates may exhibit relatively higher levels of GCs if they are frequently threatened by dominants, or if they must compete with dominants for resources.

Dominance status may also have persistent effects on levels of steroid binding globulins (Alexander and Irvine, 1998; Hattori et al., 2005; McKittrick, 1996;

Perret, 1986; Spencer et al., 1996). Binding globulins are proteins that bind to steroids in the plasma and are thought to prevent hormone diffusion through the capillaries and into tissues. Binding globulins will therefore affect both the action of steroids at tissue (through bioavailability) and their clearance rates. Despite the potential effect of binding globulins on steroid action, only a few studies have examined their role in the hormonal dynamics of dominance relationships. Many of these studies suggest that binding globulins may mediate differences in hormone action between dominants and subordinates that are otherwise undetectable when only plasma hormone levels are considered. Alexander and Irvine (1998) documented declines in CBG in subordinate horses. Though these subordinate horses showed no persistent elevation of cortisol, lower levels of CBG cause increased proportion of cortisol to be free, potentially enhancing its action. Recently, Hattori et al (2005) showed that dominant and subordinate male tree lizards (*Anolis*) do not differ in levels of total plasma testosterone, however dominant males had significantly higher levels of free (unbound) testosterone. In the lesser mouse lemur, levels of testosterone-binding globulin *increase* in subordinate males who do not breed (Perret, 1986). This leads to lower levels of free testosterone and may contribute to the subordinate's reproductive quiescence.

Differences in binding globulin capacity may also alter how the gonadal and stress hormones axes interact in dominant versus subordinate animals. A particularly interesting study by Jennings et al (2000) in the polymorphic lizard, *Urosaurus*

*ornatus*, identified a binding globulin that binds both T and corticosterone (CORT). Territorial male morphs have greater AGBG capacity than do non-territorial males. The authors hypothesize that with more CORT binding to AGBG, territorial males are less sensitive to stress-induced elevations of CORT. Thus the negative feedback of CORT on T is reduced and territorial males are able to maintain higher T levels in the face of stress. This difference in binding globulin capacity may contribute to the difference in the morphs ability to “dominate” a territory.

Binding globulins are also of particular interest in birds because, as in *Urosaurus*, they are a major point of interaction between T and GCs. In birds, both T and the GC corticosterone (CORT) bind to corticosterone binding globulin (CBG) (Deviche et al., 2001). While CBG has a much higher affinity for CORT there appears to be enough CBG present for more than 90% of T to be bound (Deviche et al., 2001; Lynn et al., 2007; Swett and Breuner, submitted). Thus, CBG is a biologically relevant regulator of plasma T. Testosterone and CORT compete for CBG binding sites such that CORT levels will in part determine how much T is bound or free (unbound) and thus affect the action and clearance of T. Therefore CBG is a major point of interaction between T and CORT, and may be involved in the interaction of these hormone systems both during and following the establishment of a dominance relationship.

In this study, we examine whether the establishment of a dominance relationship leads to persistent changes in hormones and CBG capacity in the behaviorally polymorphic white-throated sparrow. These sparrows exhibit a morph-specific difference in aggression during the breeding season and studies suggest that male morphs differ in their level of testosterone (Spinney et al., 2006; Swett and Breuner, submitted). Here we ask the following questions: do morphs differ in their ability to become dominant? Are steroid or binding globulin levels prior to pairing predictive of the future dominance status? Are there persistent changes in testosterone, corticosterone or CBG following the establishment of a dominance relationship in this species? And finally, do changes in CORT or CBG following dominance establishment alter the proportion of T that is free?

## Methods

### Study animals

The white-throated sparrow is a migratory songbird that breeds in the northeastern United States and Canada and winters in the southeastern U.S. These sparrows exhibit a unique genetic polymorphism that occurs in both sexes and is determined by the presence of a chromosomal inversion (Thornycroft, 1967; Thornycroft, 1975). Birds that bear a copy of this inversion have brighter white-stripes on the crown (WS) and are more territorially aggressive during the



breeding season (Kopachena and Falls, 1993a). Birds lacking the inversion have tan stripes on their crown and display less territorial aggression.

Wintering sparrows were captured in Travis County, Texas using seed-baited Potter traps or mist-nets. Birds were housed individually in 33 cm x 38 cm x 43 cm wire cages in an indoor aviary. Cages were separated by fabric dividers such that birds could hear, but not see each other. Birds were photo-stimulated by subjecting them to long-day photoperiods (14 light: 10 dark) for 3 weeks prior to the start of the experiment. This photoperiod mimics day lengths in the northern temperate zone during the breeding season and brings the birds into a pseudo-breeding condition. Sex was determined using a microsatellite marker on the sex chromosomes (Griffiths et al, 1998). Two observers independently determined the morph-type of each individual using plumage coloration and agreed in all cases. The proportion of white to tan in the superciliary and median crown stripes, as well as the proportion of black to brown in the lateral crown stripes were considered. This protocol is roughly based on the method of Piper and Wiley (1989).

#### Experimental procedure

Prior to pairing, one bird in each pair was marked with a black-plastic leg band for easy identification during behavioral observations. We alternated which morph (WS or TS) was marked in each pair. Birds were bled either three days

prior to pairing (2 pairs in 2002), or immediately prior to pairing (6 pairs in 2003). 100  $\mu$ L of blood was drawn via venipuncture of the alar vein. Blood was collected in a heparinized microhematocrit tube via capillary action. Birds were bled within 3 minutes of the experimenters entering the room in which birds were housed.

Eight pairs were formed, each consisting of one WS male and one TS male. Pairs were housed in a 33 cm x 38 cm x 43 cm cage, and cages were separated from each other by fabric dividers. Thus pairs were visually isolated from other birds in the room (these included other paired males, singly housed males and females.) Each cage contained a single covered food dish (which allowed only one bird to feed at a time) and two water dishes. Food was given ad libitum, so while food amount was not limited, access to food could be controlled by the dominant bird. Following pairing (on day 1) birds were videotaped for 20-30 minutes on days 1, 2 and 3 (two pairs in 2002) or on days 2, 3 and 4 (six pairs in 2003.)

One of the experimenters or an independent observer watched the videos and scored the following behaviors: displacement, chasing, attack, threat and singing. Additional behaviors were scored in the 6 pairs recorded in 2003; these included: feeding, drinking, bill wiping, crown raising, body feather puffing, tail wagging, preening and bathing. Observers were essentially blind to the morph of the bird as plumage details were difficult to see in the video footage. For a

description of behaviors see Table 1. We chose four of the most clearly aggressive behaviors: attacks, threats, trills and displacements, to calculate a dominance score. Each time a bird exhibited one of these behaviors it received one point. Points for each bird were summed and the sum was corrected for the number of minutes of observation. The bird in each pair who earned the highest score was designated the dominant bird.

Four days following pairing, birds were bled again following the procedure described above. All blood samples (pre- and post-pairing) were centrifuged and plasma was drawn off using a Hamilton syringe. Plasma was then frozen at approximately -20°C until it could be assayed.

#### Hormone and Binding Globulin Assays

Testosterone: White-throated Sparrow plasma testosterone levels were measured using an enzyme immunoassay kit from Assay Designs (cat # 901-065). The assay protocol was optimized for this species as described in Swett and Breuner (submitted.) Individual plasma samples were thawed, picofuged, vortexed and diluted with assay buffer to a 1:20 concentration. Samples were run in triplicate. The standard curve (6 standards ranging from 2,000 pg/mL to 8.2 pg/mL) and a separate external standard were also run in triplicate on each plate. Conjugate-bound T and antibody were added and the plate was incubated at 26°C on a shaker for 2 hours. Wells were then emptied and rinsed with wash

buffer and enzyme substrate was added. The plate was incubated for one hour, again at 26°C but without shaking. Following the second incubation, stop solution was added to each well and the plate was immediately read using a Multiskan Ascent microplate reader at 405 nm corrected at 595 nm. The lower limit of detectability ranged from 0.8 to 2.9 pg/well. Samples from each morph and time point were randomly distributed across plates.

Corticosterone: Plasma CORT was also measured using an Assay Designs corticosterone enzyme immunoassay kit (cat # 901-097) following a similar protocol optimized for use in the congeneric White-crowned sparrow and then checked for accuracy in the white-throated sparrow (for detailed description of protocol and optimization see Wada et al, 2007). Plasma was diluted 1:40 with assay buffer and a 1% concentration of steroid displacement buffer was used. Again, morphs and pre- vs. post-interaction samples were randomized across plates.

CBG: Previous work characterized the binding affinity and specificity for CBG in white-throated sparrows (described in Swett and Breuner , submitted.) White-throated sparrow plasma CBG binds both CORT and T at a single binding site, but with 7-fold difference in affinity (CORT EC<sub>50</sub> =  $4.17 \pm 0.30$  nM; T EC<sub>50</sub> =  $2.82 \pm 0.24$  nM).

Individual CBG capacity in all samples was assayed with a point sample analysis using one near-saturating concentration of CORT, allowing for estimation of total capacity based on the percent bound equation:  $\% \text{ bound} = \frac{[\text{ligand}]}{[\text{ligand}] + [\text{dissociation constant}]}$ . First, plasma was stripped of endogenous CORT via incubation with dextran-coated charcoal solution (0.1% dextran, 1% norit A charcoal in 50mM Tris) for 30 minutes at room temperature. Samples were centrifuged and supernatant removed. Each sample was incubated on ice at a final dilution of 1:1089 for 2 hours with 20 nM [ $\text{H}^3$ ] CORT and 1  $\mu\text{M}$  unlabeled CORT to determine non-specific binding. This concentration of radio-labeled ligand was to occupy approximately 91% of binding sites (based on a dissociation constant of 1.595 Swett and Breuner, submitted). Immediately following incubation, plasma solutions were passed via rapid vacuum filtration over PEI (0.3%) soaked Whatman GF/B paper filters. Filters were rinsed three times with 3 mL ice cold 25mM Tris.

Radioactivity bound to filters was quantified via standard liquid scintillation spectroscopy. Briefly, filters were allowed to dry, placed in scintillation vials, 100  $\mu\text{L}$  isopropyl alcohol and 5 mL scintillation fluid were added (Scint Safe 50%, Fisher or Perkin Elmer Ultima Gold.) Radioactivity was measured in a Beckman-Coulter LS 6500 Multi-purpose Scintillation counter or a Perkin Elmer Tri-Carb 2900TR scintillation counter.

Free testosterone: Free testosterone was calculated as in Lynn et al, (2007), Zysling et al. (2006) and Deviche et al (2000). First the amount of CBG occupied by CORT was determined and subtracted from the total capacity (CBG has a 7-fold greater affinity for CORT than T, and so will preferentially bind CORT). The remaining CBG was then used to figure free T levels based on the equation in Barsano and Baumann (1989). There is currently no mathematical equation describing the binding of two hormones to a globulin at once; additionally, the free levels of T are so low it is nearly impossible to measure with a direct assay in such a small animal (<30 grams). As such, these methods are the best available way to estimate unbound testosterone.

### Statistical Analyses

Data was log or ln transformed where appropriate to normalize sample distribution. Pairs run in both years (2003 and 2004) were analyzed together. Though the dominant birds in 2002 had significantly higher dominance scores, we are analyzing the relationship between endocrine measures and the categorical variable “status”, not the numerical dominance score. Therefore this difference between years is not relevant. Total T, total CORT, CBG and free T data were analyzed using repeated measures ANOVA with individual as the random effect. These analyses were performed using JMP 5.0.1a.

### Results

### Dominance Behavior

According to behavioral scoring, WS males dominated TS males in 6 of 8 pairs (Fisher's exact test, 2-tail,  $p=0.13$ ). Means and SE of all observed behaviors are given in Table 2. Morphs differed significantly only in number of displacements. WS males displaced TS males significantly more often than vice versa (Fig 3:  $F=9.3$ ,  $p=0.009$ .) Only one TS male performed threat displays compared to 5 out of 8 WS males, however the mean number of threat displays per 60 minutes did not differ between morphs. Attacks were rare and only observed 3 times in 9 hours of observation making it difficult to detect an effect of morph statistically. However, both birds that performed attacks were WS males. Morphs did not differ in song rate ( $F=0.37$ ,  $p=0.55$ .)

### Effect of Social Status on T, CORT, CBG and free T

Dominant and subordinate males did not differ in levels of total T, total CORT, CBG or free T prior to pairing thus these measures were not predictive of future status (Fig. 1). Nor did dominants and subordinates show persistent alterations in hormone levels in response to the change in social environment. Social status had no significant effect on post-pairing measures of total T, total CORT, CBG or free T (total T:  $F=0.98$ ,  $p=0.51$ ; total CORT:  $F=0.86$ ,  $p=0.61$ ; CBG:  $F=0.62$ ,  $p=0.82$ ; and free T:  $F=0.22$ ,  $p=0.99$ .)

### Effect of Morph on T, CORT, CBG and free T

Morphs did not differ in plasma levels of total T ( $F=0.97$ ,  $p=0.52$ ), total CORT ( $F=0.86$ ,  $p=0.61$ ), CBG ( $F=0.62$ ,  $p=0.82$ ) or free T ( $F=0.21$ ,  $p=0.99$ ) either before or after pairing, nor did they differ in response to pairing.

### Discussion

#### Difference in dominance ability between morphs

WS males tended to dominate TS males (6 out of 8 pairs), however this trend was not significant. Given that WS males exhibit greater territorial aggression, we had predicted that they would dominate TS males. Previous studies of dominance in this species have shown that morphs do not differ in social status when birds are held under a short-day photoperiod. However this photoperiod mimics the non-breeding season when birds associate in flocks. Dominance relationships may differ during the breeding season, when birds are territorial and males exhibit relatively higher levels of aggression. Only one other study has evaluated the relative dominance ability of morphs under a long-day photoperiod. Houtman and Falls (1994), using round-robin tournament style competition trials, found that although TS birds occasionally dominated WS birds, WS birds were



significantly more likely to be dominant. We suspect that a lack of power prevented us from detecting a statistically significant difference in dominance ability between the morphs. Had our sample size been 16 pairs, rather than 8, and the same proportion of WS males had become dominant, we would have reached a significance level of  $p=0.01$  (Fisher's Exact Test.)

#### Difference in behavior between morphs

Displacement was the most common dominance behavior. Across pairs, WS males displaced TS males significantly more frequently than the reverse. More intense aggressive behaviors such as attacks and threats were less frequent and only WS males performed these behaviors save for a single TS male who displayed threats. Interestingly, there was no difference between morphs in the frequency of song, though WS males tend to sing more than TS males during the breeding season (Falls and Kopachena, 1994). Previous studies of captive, short-day photoperiod sparrows have observed no differences in song frequency between the morphs. It is possible that captive birds lack the appropriate environmental cues to elicit a song rate similar to that of free-living birds. Song was markedly less frequent among our captives compared to birds observed in the field (personal observation). Overall, we conclude that WS males exhibit more frequent and intense aggression. This behavior likely contributes to their tendency to acquire dominant status.

### Relationships between Social Status and T, CORT, CBG and free T

Pre-pairing T, CORT, CBG or free T did not differ between males who would become dominant in their dyad versus those that would become subordinate. Thus, none of these parameters were predictive of dominance ability. The lack of relationship between pre-pairing T and future rank is consistent with the findings of Schwabl et al's (1988) study on captive winter flocks of white-throated sparrows. Schlinger (1987) did find a nonsignificant ( $p=0.10$ ,  $N=10$ ) correlation between autumn androgen levels and aggression observed over the winter.

Neither total T, or total CORT showed persistent differences between dominants and subordinates. In addition there were no substantial changes in levels of CBG following dominance establishment, and so free T levels mirrored those of total T and were not significantly different between dominants and subordinates post-pairing. Once a dominance relationship or social hierarchy is stable, levels of aggression typically decline and dominants and subordinates may have similar levels of T (Stelkis, 1986; Schwabl et al., 1988). Intense aggressive behaviors such as attack or threat displays were infrequent in this study. Aggressive conflict between males may have been more intense had potential mates been available. Females were housed in the same room as males both prior to and following pairing, however males were visually isolated from females at all times.

The fact that we do not see an elevation of CORT in subordinates relative to dominants post-pairing suggests that these birds did not perceive their social subordination as stressful. Goymann and Wingfield (2004) argue that the costs of being subordinate (or dominant) will determine the allostatic load associated with that status and that this load determines whether glucocorticoids will be elevated. According to the criteria laid out in their paper, we would expect our subordinate sparrows to have a higher allostatic load and higher levels of CORT than dominants. Subordinates in our study were regularly subjected to low-level aggression (displacement), and were observed to pant more frequently which may be an indicator of stress. Subordinates had no means of avoiding dominant birds, and had to share a food dish (resource) with them. Perhaps the sparrows did not perceive this as competition for food as the dish was refilled ad libitum. Also subordinates may not have felt sufficient threat due to the infrequency of intense aggression (i.e. threats or fights).

Unlike the studies in horses (Alexander and Irvine, 1998) and rats (McKittrick, 1996; Spencer et al., 1996), we did not see a decline in CBG in subordinate sparrows. In both rats and horses, the aggression displayed by dominants was intense, thus the social stress of subordination may have more severe than in our study. It is possible that social stress in our experiment was not severe enough to lead to persistent changes in CBG. Our results suggest that in the white-

throated sparrow, the effect of social status on T and CORT may not be modified by binding globulins.

#### Effect of Morph on T, CORT, CBG and free T

We observed no difference between male morphs in pre-pairing levels of T, CORT, CBG or free T. Nor did we see any difference between morphs in how these parameters changed following dominance establishment.

The lack of difference between morphs in pre-pairing total T levels is contrary to recent findings in wild-caught sparrows (Spinney et al., 2006; Swett and Breuner, submitted). Though we subjected our males to long-day photoperiods that mimic what they experience in the wild, there are many other environmental cues that were absent from our aviary; these cues may be necessary for eliciting full activation of the reproductive axis. As we have previously observed in wild-caught breeding sparrows, baseline CORT and CBG did not differ significantly between morphs, hence there was also no significant difference in free T. Neither morph showed significant changes in CBG, CORT, T or free T in relation to its social status. Thus the morphs do not differ in their endocrine response to the social interaction. The tendency for WS males to dominate TS males in our experimental scenario cannot be attributed to any persistent endocrine response to social challenge. Morphs may differ in their short-term, transient endocrine

responses to social challenge and it would be useful to evaluate this possibility both in captivity and in the field.

### Conclusions

WS males exhibited a greater frequency and intensity of aggression and this led to their tendency to dominate TS males in captivity. Morphs did not differ in either their pre-interaction levels of total T, free T, CORT or CBG, nor did they show persistent changes in these endocrine measures once a dominance relationship was established. In addition, we found no evidence of prolonged changes in total T, free T, CORT or CBG when sparrows assumed either a dominant or subordinate social status. We conclude that, if they occur, changes in these endocrine measures would be confined to the brief period of social instability during dominance establishment. In this study, once the dominance relationship was stable, the social stress associated with being subordinate was likely minimal.

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## Endocrine Measures in Relation to Morph

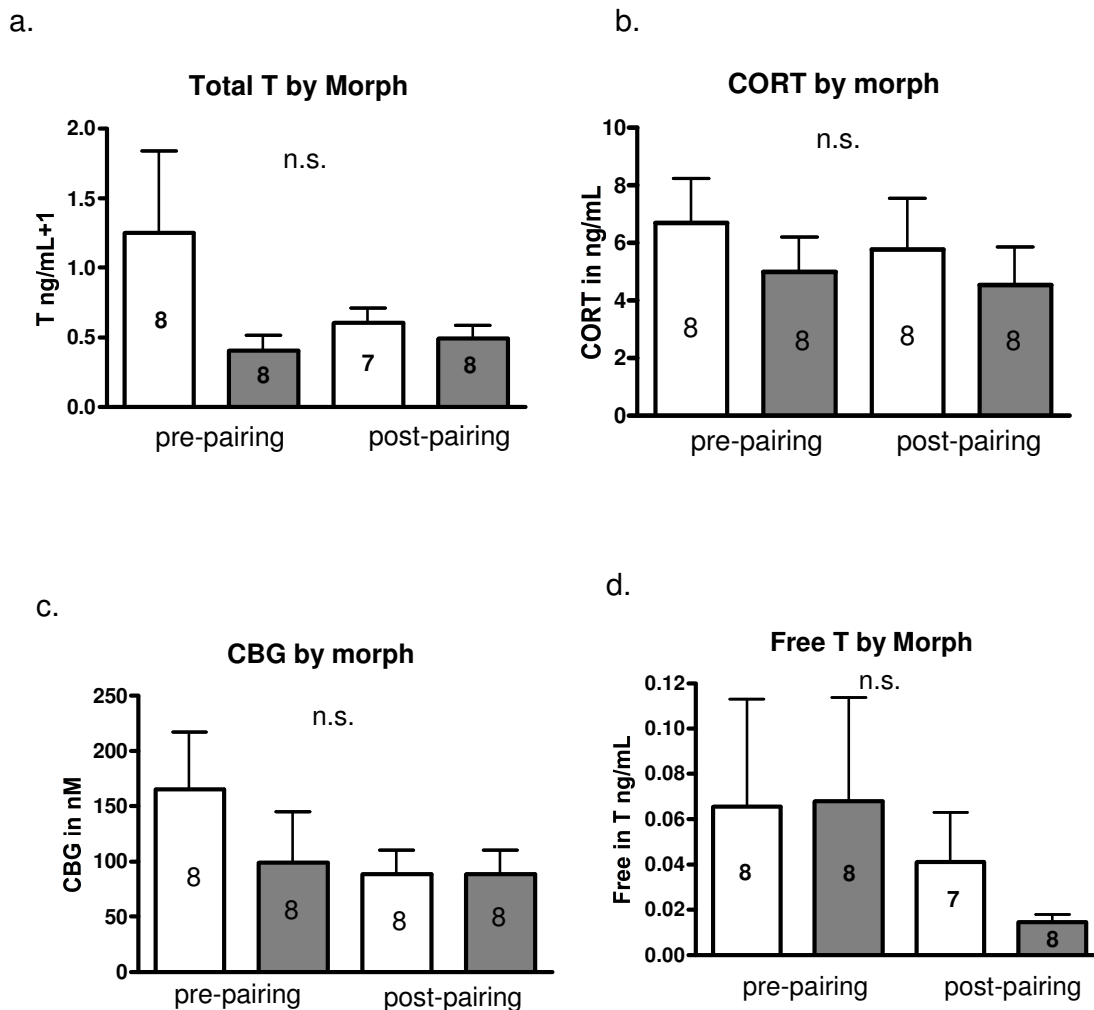


Figure 1. Total T (a), total CORT (b), CBG (c) and free T (d) in WS (white bars) and TS (gray bars) males both before and after establishment of a dominance relationship. Sample sizes are indicated in the bars. There were no significant differences between morphs either before or after pairing; nor did morphs differ in their response to the establishment of a dominance relationship. Total T ( $F=0.97$ ,  $p=0.52$ ), total CORT ( $F=0.86$ ,  $p=0.61$ ), CBG ( $F=0.62$ ,  $p=0.82$ ), or free T ( $F=0.21$ ,  $p=0.99$ .)

## Endocrine Measures in Relation to Social Status

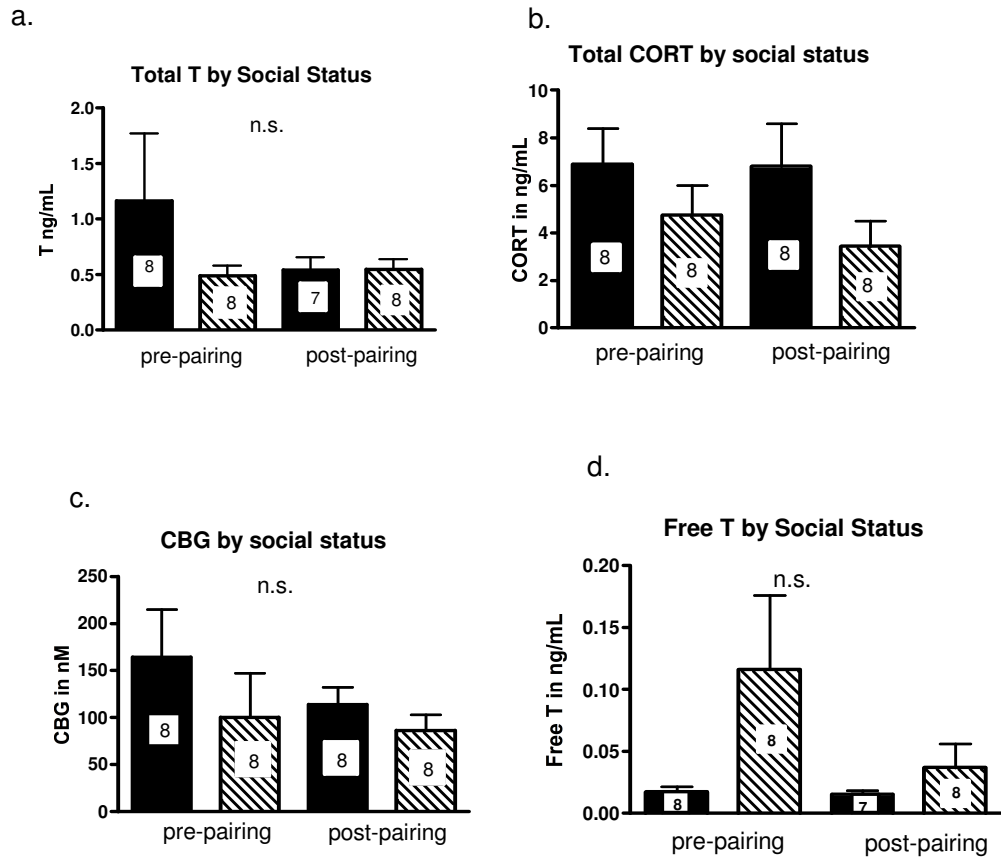


Figure 2. Total T (a), Total CORT (b), CBG (c) and free T (d) in dominant (black bars) and subordinate (hatched bars) males before and after establishment of a dominance relationship. Sample sizes are indicated in bars. These endocrine measures did not differ significantly between dominants and subordinates either before or after pairing; nor did dominants and subordinates differ in their response to the establishment of the dominance relationship. Total T ( $F=0.98$ ;  $p=0.51$ ); total CORT ( $F=0.86$ ,  $p=0.61$ ); CBG ( $F=0.62$ ,  $p=0.82$ ) and free T ( $F=0.22$ ,  $p=0.99$ .)

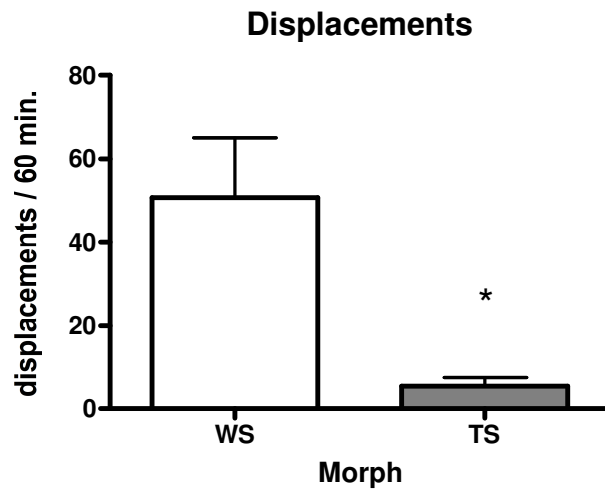


Figure 3. Mean displacements performed by WS (white bar) and TS males (gray bar) in 60 minutes. WS males displaced TS males significantly more often ( $F=9.3$ ,  $p=0.009$ ,  $N= 8$  WS and 8 TS)

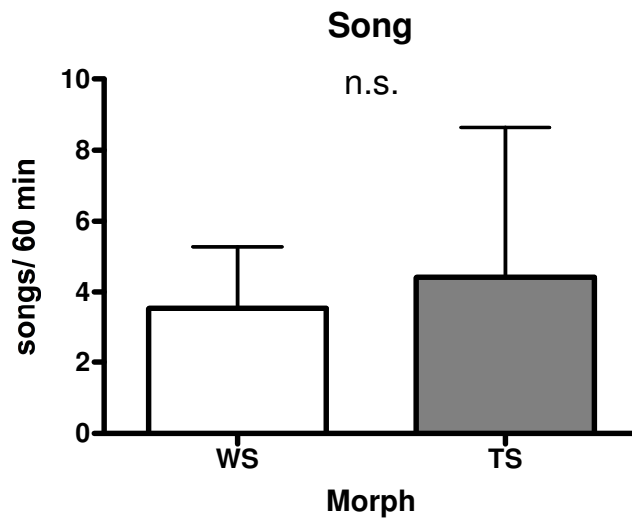


Figure 4. Mean number of songs given in 60 minutes by WS (white bar) and TS (gray bar) males. There was no significant difference between morphs ( $F=0.37$ ,  $p=0.55$ ,  $N= 8$  WS and 8 TS).

**Table 1. Descriptions of Behaviors**

Tail wag	Side to side movement of tail.
<b>Behavior</b>	<b>Description</b>
Displacement	<b>Criteria:</b> 1. "Displacer" initiates movement before the "displacee" moves. 2. "Displacer" assumes location that is within one body length of "displacee's" original location. 3. Interaction involves only one change of position by the "displacer".
Threat behavior in Zebra Finches	<b>Criteria:</b> 1. "Chaser" initiates chase by either moving first or indicating intention to move by body position. 2. "Chaser" ends chase by being the first to stop flying. 3. Chase involves more than one change of location and the interval between one change of location and the next is essentially immediate.
Threat	<b>Criteria:</b> 1. The bird that is threatening points its body towards the threatened bird and tracks that bird's movements with its head. 2. Bird that is threatening MAY vocalize a trill or flutter its wings, however the trill and flutter are not necessary for the behavior to be considered a threat. 3. The threatening bird indicates aggression to the threatened bird through body posture without necessarily moving towards or otherwise actively disturbing the threatened bird Our definition of threat behavior is based on the head-forward D1 display ((Senar, 1990)) and threat behavior in <i>Taeniopygia guttata</i> (Figueredo et al., 1992).
Singing	Count of complete songs
Feeding	Count of number of seeds picked up and hulled
Drinking	Count of number of times bill dipped into water dish
Panting	Count of panting bouts; bouts separated by 30 pause or change of activity
Bill wiping	Count of wiping bouts; bird must pause and lift head between bouts
Preening	Count of preening bouts with a pause of 30 seconds or change of location between bouts.
Crown raise	Feathers on crown erected
Puff Body	Erection of body coverts

**Table 2. Summary statistics for all behaviors observed during study**

	displacement	threat	panting	Sing	chase	bill wipe	crown raise	puff body	tail wag	attack	feed	drink	preen
<b>WS</b>	<b>50.75</b>	<b>12.63</b>	<b>1.67</b>	<b>3.54</b>	<b>0.33</b>	<b>41.5</b>	<b>7.33</b>	<b>28.5</b>	<b>10.83</b>	<b>0.291</b>	<b>48.17</b>	<b>11.33</b>	<b>0.67</b>
<b>Mean <math>\pm</math> SE</b>	$\pm$ 14.27	$\pm$ 5.95	$\pm$ 1.67	$\pm$ 1.74	$\pm$ 0.33	$\pm$ 17.9	$\pm$ 0	$\pm$ 7.9	$\pm$ 4.98	$\pm$ 1.93	$\pm$ 16.24	$\pm$ 3.52	$\pm$ 0.49
<b>TS</b>	<b>5.46</b>	<b>4.38</b>	<b>1.83</b>	<b>4.41</b>	<b>1.5</b>	<b>37</b>	<b>15.17</b>	<b>19.67</b>	<b>4.33</b>	<b>0</b>	<b>23.33</b>	<b>6.33</b>	<b>0.5</b>
<b>Mean <math>\pm</math> SE</b>	$\pm$ 2	$\pm$ 4.38	$\pm$ 0.87	$\pm$ 4.23	$\pm$ 1.5	$\pm$ 14.35	$\pm$ 8.74	$\pm$ 8.79	$\pm$ 3.74	$\pm$ 0	$\pm$ 9.17	$\pm$ 2.51	$\pm$ 0.34
<b>F, p-value*</b>	7.21, 0.02	1.25, 0.28	2.55, 0.2	0.08, 0.80	0.57, 0.47	0.68, 0.43	0.33, 0.59	0.91, 0.36	2.78, 0.15	2.27, 0.15	1.07, 0.34	0.07, 0.8	0.1, 0.78
<b>Total # of observations</b>	<b>449.67</b>	<b>136</b>	<b>21</b>	<b>63.67</b>	<b>11</b>	<b>471</b>	<b>135</b>	<b>289</b>	<b>91</b>	<b>2.33</b>	<b>429</b>	<b>106</b>	<b>7</b>

\*Data were log transformed where appropriate prior to ANOVA testing.